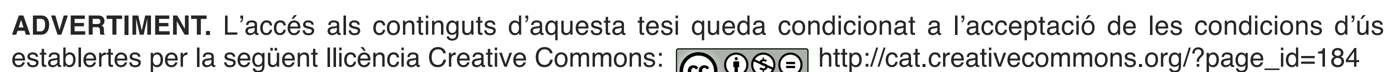
ADVERTIMENT. L'accés als continguts d'aquesta tesi queda condicionat a l'acceptació de les condicions d'ús establertes per la següent llicència Creative Commons:  http://cat.creativecommons.org/?page_id=184

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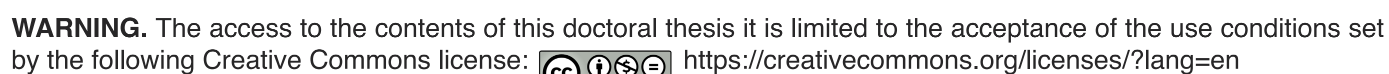
WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license:  https://creativecommons.org/licenses/?lang=en

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Ultraviolet light (UV-C) as a redundant biosafety step for pathogen inactivation in the manufacturing process of spray dried plasma from animal origin.

Tesi doctoral presentada per **Elena Blázquez Salvador** per accedir al grau de Doctora en el marc del Programa de Doctorat en Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció dels Doctors **Francisco Javier Polo Pozo**, **Joaquim Segalés i Coma**, i **Joan Pujols i Romeu.**

Bellaterra, 2019

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Certifiquen:

Que la tesi doctoral titulada "**Ultraviolet light (UV-C) as a redundant biosafety step for pathogen inactivation in the manufacturing process of spray dried plasma from animal origin**" presentada per Elena Blázquez Salvador per a l'obtenció del grau de Doctora Medicina i Sanitat Animals, s'ha realitzat sota la seva supervisió i tutoria, i autoritzen la seva presentació per a que sigui valorada per la comissió establerta.

I per tal que consti als efectes oportuns, signen el present certificat a Bellaterra a 12 de març de 2019.

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A la meva família

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Chapter 1

Introduction

1.1 Spray-dried Plasma

Spray dried plasma (SDP) is a functional protein source obtained from blood of healthy animals, approved to be sacrificed for human consumption after veterinary inspection. Blood of these animals is collected at the slaughterhouse, treated with an anticoagulant, chilled and transported to industrial facilities in which blood is centrifuged to separate the red blood cells (RBC) from the plasma fraction. Plasma is then concentrated either by membrane filtration or vacuum evaporator and spray dried at high temperatures (80ºC throughout its substance) to convert it in a powder. Such material preserves the biological activity of its proteins, mainly albumins and globulins, being immunoglobulin G (IgG) the predominant antibody. The SDP can be obtained from pigs (SDPP) or bovine (SDBP) and can be used for human foodstuff and animal feeds (Howell and Lawrie, 1983; Gatnau *et al.*, 1989).

1.2 Uses of spray-dried plasma

SDP has been used as a functional protein source included in piglets feed diets since the late 1980s (Zimmerman, 1987; Gatnau *et al.*, 1989). SDP is mainly used in pig feed diets at an inclusion level between 4-8% (Coffey and Cromwell, 2001; Van Dijk *et al.*, 2001; Torrallardona, 2010) to significantly improve daily gain, feed intake, production efficiency, morbidity indices and piglet survival (Coffey and Cromwell, 2001; Van Dijk *et al.*, 2001; Torrallardona, 2010). SDP also help reducing post-weaning lag caused by the appearance of post-weaning diarrhea (Gatnau and Zimmerman, 1991; van der Peet-Schwering and Binnendijk, 1995; Cain and Zimmerman, 1997).

The active biological fraction of the SDP involved in these improvements in the animals appears to be the immunoglobulin rich fraction of plasma (Pierce *et al.*, 2005), although there is controversy about the key proteins or bioactive peptides present in plasma fraction responsible of the health benefits associated with its use. This may explain why bovine plasma has a similar effect than SDPP.

Pigs are weaned before being able to synthesize their own immunoglobulin A (IgA) (Svendsen and Brown, 1973; Pérez-Bosque *et al.*, 2016), so post-weaning diets that include plasma are beneficial, as the presence of immunoglobulins contributes to the defense against infections, opsonizing pathogens and neutralizing toxins (Svendsen and Larsen, 1977). In this way the SDP helps to strengthen the immune system and acting against pathogens (Coffey and Cromwell, 1995; Bergström *et al.*, 1997). In addition to immunoglobulins, SDP contains functional proteins like cytokines, growth factors, bioactive peptides, amino acids and other biological components that may contribute to the health improvements observed when administered to animals (Lallès *et al.*, 2009). These proteins can interact with immune cells present in the intestinal mucosa thus changing the cytokine environment (Pérez-Bosque *et al.*, 2016).

1.3 Industrial production of spray dried plasma

The SDP production at industrial level is carried out following the good manufacturing practices (GMP) and the highest quality standards in order to produce a high-quality product. Such product must be safe for its use in human food and animal feed and must guarantee the

stabilization of its active components. The production process of the SDP consists of several stages that take place in different locations, as indicated below.

1.3.1 Blood collection at the abattoir

Only the blood from healthy animals, certified by the competent state authority as suitable to be sacrificed for human consumption, is collected at the slaughterhouse. The blood is collected in a stainless steel pan in which anticoagulant is gradually added, usually sodium citrate (Coffey and Cromwell, 2001) or sodium tripolyphosphate (Rangel *et al.*, 1995), to prevent blood clotting.

The blood collection system is separated from the rest of the animal processing chain, and the whole manufacturing process from the time that the blood is collected to the final packed product, is conducted in close circuit avoiding the possibility of cross contamination with other tissues or from the external environment. In the USA, the collection system in the slaughterhouse pours the blood in an industrial centrifuge to proceed to the separation of RBC and plasma. After plasma separation, this liquid fraction can be concentrated or refrigerated (without concentration) to 4ºC and transported to the processing plant. In contrast, in Europe the collection system spills the blood in stainless steel containers and these are kept refrigerated at 4ºC waiting for the subsequent transport to the processing plants.

Once the daily blood collection is completed, the entire collection circuit is cleaned by a CIP (cleaning in place) process consisting of a first cleaning with water, followed by the use of chemical products and heat, to ensure proper sanitization of the circuit and, finally,

a rinse with water to eliminate the remnants of chemicals that may have remained.

1.3.2 Transport to the processing plants

Blood or plasma collected at slaughterhouse stored in batches within refrigerated containers is transported in refrigerated trucks that are sealed after filling. Each batch of blood comes from about 6,000 – 7,000 pigs or 1,500 – 2,000 cattle and each batch of plasma comes from about 10,000 - 11,000 pigs or 2,500-3,000 beef cattle. To make a correct traceability of the product, each lot is identified with the name of the slaughterhouse and its location, date of collection of the product, truck used for transport, identification of the driver and quality of the product collected.

Once the transport truck arrives at the processing plant, an inspection of the color of the product is made by measuring the absorbance at 400 nm that is correlated with a color scale from 0 to 4 depending on the lysis of the hemoglobin; the higher the absorbance of the plasma, the higher value in the scale. Plasma for commercial use in Spain should not be higher than 3. A higher value of the color scale would indicate more hemolysis of the blood, which would affect the color of the final product. An olfactory inspection of the product is also carried out, as well as a temperature measurement, to declare the product suitable for processing. If the temperature of the product exceeds 10ºC, implies a failure in the maintenance of the cold chain, which is declared not suitable for the production of SDP.

Once the product has been certified as suitable for the production of SDP, the content of the truck is deposited in a refrigerated industrial tank to begin with the production of the SDP. When the truck

is completely emptied, a CIP process in the truck is carried out to assure the cleaning and disinfection of the cube before the next collection.

1.3.3 Processing and spray-drying

In the first stage of the SDP production process, the blood deposited in the tank is taken through a closed circuit to an industrial centrifuge, to separate the plasma from the RBC. The RBC are stored for the subsequent production of other products while the plasma is diverted through the circuit to the filtration zone, where the plasma is concentrated by vacuum evaporator or by filtration membranes either by nanofiltration or reverse osmosis. Once concentrated, the plasma is pumped into the industrial spray-dryer, which will convert the concentrated liquid plasma into powder.

Spray-drying consists of the desiccation of a liquid solution or suspension into a dried particulate powder by spraying the feed into a hot drying chamber (see figure 1). The spray-drying process involves four stages of operation that affect microbial survival and the characteristics of the resulting product: 1) atomization of liquid source in form of particles with a diameter of 45 – 150 µm into a hot chamber; 2) contact between the spray and the drying medium consisting of a very hot air, between 170 – 310 ºC (Pérez-Bosque *et al.*, 2016), at a high gas mass to liquid mass flow volume ratio; 3) moisture evaporation resulting in particle formation; and 4) separation of dried products from the air stream (Cal and Sollohub, 2010; Kuriakose and Anandharamakrishnan, 2010; Sollohub and Cal, 2010) following a residence time between 20 – 90 seconds. During spray-air contact, droplets interact with the hot air in

the spraying chamber. Initially, as moisture is lost, the particle is maintained at the adiabatic wet bulb temperature, then, the droplet temperature increases to reach a value close or similar to the outlet air temperature (Straatsma *et al.*, 2007; Perdana *et al.*, 2015). Inlet and outlet temperatures are the two main parameters that have a major influence on the inactivation of microorganisms. The European Animal Protein Association (EAPA) and the North American Spray Dried Blood and Plasma Producers (NASDBPP) have established more than 170ºC for the inlet temperature and 80-84ºC for the outlet temperature for SDP industrial manufacturing. Inactivation occurs predominantly during the initial period of drying, while the remaining drying time further decreases moisture content. The survival of microorganisms is reduced by increasing the inlet temperature, but the outlet air temperature has the greatest impact on pathogen inactivation because this is the minimum temperature that the particle will achieve during the drying process; therefore, the higher outlet temperature, the higher microbial inactivation (Perdana *et al.*, 2013, 2015). Relatively high drying temperatures, rapid changes in temperature and pressure, and rapid dehydration are the phenomena involved in microbial inactivation. Dehydration causes damages in the cells, mainly in the cytoplasmic membrane (Crowe *et al.*, 1987; Lievense and Van't Riet, 1994) and also produces damage to DNA/RNA and proteins (Lievense *et al.*, 1992) causing significant effects on microorganism survival (Lievense *et al.*, 1992; Lievense and Van't Riet, 1994; To and Etzel, 1997a, 1997b; Ananta *et al.*, 2005; Dobry *et al.*, 2009; Perdana *et al.*, 2013).

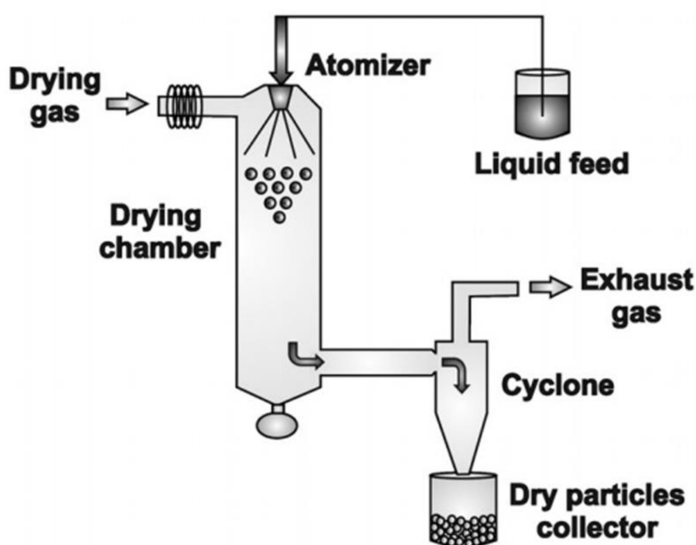


Figure 1: Typical spray-dryer system. The liquid solution is sprayed into the drying chamber. Contact with hot air at 170-310ºC produces a liquid dehydration, resulting in powder formation. From: Sosnik and Seremeta, 2015**.**

1.3.4 Scale up from laboratory dryers to industrial dryers

Laboratory spray-dryers are useful for establishing guidelines to scale up the industrial production of SDP. Nevertheless, differences between the process carried out in both scales have been described (Thybo *et al.*, 2008). The main differences between laboratory and pilot plant dryers compared to industrial dryers are design, size and volume processed, all of which affect the retention or dwell time of the product within the chamber (Foster and Leatherman, 1995). Laboratory spray-dryers have reduced a retention or dwell time of the product within the chamber (<1 sec) compared with commercial driers (between 20 and 90

seconds, depending upon scale and design of the dryer). Furthermore, there is an immediate cooling to room temperature of the small quantity of dried product produced by lab-dryers in comparison with industrial dryers, which process much larger quantity of material that extends the time for dissipation of heat from the dried product. It has to be taken into account that commercial dryers may be more effective than laboratory driers to inactivate microorganisms as suggested by Perdana et al. (2013).

1.3.5 Packaging, storage, traceability and quality control

As SDP is being produced, the powder is stored in a small silo. From the silo, SDP is directly bagged in packs. Each pack contains a code with information about production lot, production plant, date of packaging, operators and drying line. This coding system allows the identification and traceability of all manufactured products. The SDP packed final product is normally stored at room temperature (≥ 20ºC) at least for 14 days before proceeding to its sale.

1.4 Biosafety steps in the production process of SDP

SDP, as a product obtained from fresh blood, is a raw material that requires the presence of several safety steps in its production process to eliminate any potential microbiological risk from the raw material. The biosafety of SDP is often questioned, particularly during periods of emergence or re-emergence of swine or bovine diseases. There are

numerous safety features in the industrial manufacturing process of SDP that acting together, allows obtaining a safe final product.

The first biosafety step and critical control point in the production process of SDP is the fact that only the blood from healthy animals certified as fit for slaughter for human consumption is collected. Usually, blood from healthy animals is considered sterile; however, it may be cases in which the blood from an animal with an asymptomatic disease and/or with a viremia, is collected. For that reason, the plasma production process must have at least one specific step of pathogen inactivation.

The second biosafety step in the production process of SDP is the pooling of blood to obtain the pooled plasma, from about 10,000 - 11,000 pigs or 2,500 - 3,000 beef cattle. The pooled plasma inherently contains neutralizing antibodies against numerous habitual pathogens; therefore, neutralizing antibodies contributes to the biosafety of the final product (Solheim and Seghatchian, 2006; Williams and Khan, 2010; Polo *et al.*, 2013).

The next biosafety step in the production process of SDP is the spray-drying process. During such process, computer systems designed to control and monitor processing temperatures and conditions are used to ensure that SDP has been exposed to a minimum of 80ºC throughout its substance. This is one of the most important critical control points in the manufacturing process of SDP.

So many experiments of artificial inoculation of pathogens in plasma have demonstrated that spray drying is a very effective technology to inactivate important pathogens of interest in the swine industry as *Salmonella enterica* (Polo *et al.*, 2002; Annex 2)*, Porcine reproductive and respiratory syndrome virus* (PRRSV) (Polo *et al.*,

2005), *Pseudorabies virus* (PRV) (Polo *et al.*, 2005), *Swine vesicular disease virus* (SVDV) (Pujols *et al.*, 2007) *and Porcine epidemic diarrhea virus (PEDV)* (Gerber *et al.*, 2014; Pujols and Segalés, 2014). Furthermore, other studies demonstrated lack of transmission of different viruses of swine, as *Porcine circovirus 2* (PCV-2), one of the most thermal resistant virus of swine (Welch *et al.*, 2006; Opriessnig *et al.*, 2010), when pigs were fed with SDPP containing genome copies of PCV-2 (Opriessnig *et al.*, 2006; Pujols *et al.*, 2008, 2011; Shen *et al.*, 2011). Also, a retrospective study of different SDPP samples and sera from pigs fed with SDPP collected over time, showed that SDPP containing RNA and antibodies of *Hepatitis E virus* (HEV) did not transmit HEV to pigs (Pujols *et al.*, 2014).

Another important critical control point once the SDPP has been produced is the storage conditions. SDPP is a dry product with low moisture (<9%) and very low water activity (a_w<0.6). Some pathogens, especially bacteria and enveloped viruses, are not able to survive for prolonged periods of time in dry materials like SDPP (Sampedro *et al.*, 2015). Several mechanisms affecting microbial survival in dry materials have been described, such as, oxidative stress and reactive oxygen species formation, which produces lipid peroxidation, and the browning reaction of sugars causing protein denaturation and DNA damage. These changes are accumulative and have lethal effects on bacterial metabolism (Hernández-García, 2011). Therefore, as an additional safety feature, most manufacturers package and store porcine SDPP at room temperature (>20°C) for at least 14 days before release for sale. These storage conditions have been demonstrated as effective to inactivate certain pathogens susceptible to dry environments and mild

temperatures, such as PRRSV, PEDV and coronaviruses in general (Pujols and Segalés, 2014; Sampedro *et al.*, 2015).

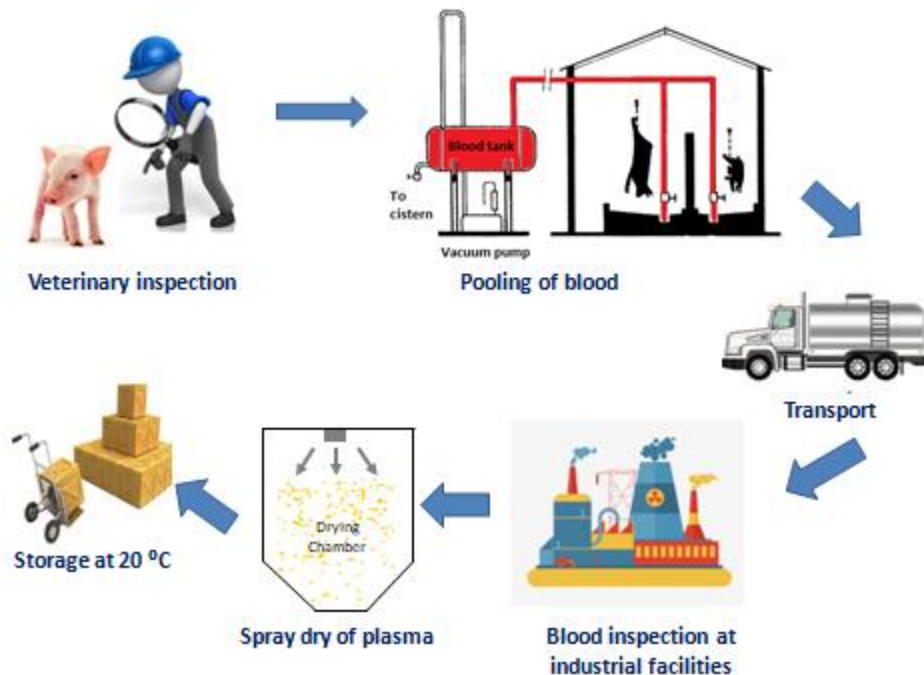


Figure 2: Biosafety steps in the production process of SDP. Veterinary officers inspect the animals and only those declared fit for slaughter enter into the food chain. Their blood is collected, pooled and transported to industrial facilities, where the SDP will be produced and stored for 14 days at >20ºC until its release.

1.5 Regulatory status of SDP

SDP is classified as an animal by-product. The elimination of animal by-products from the meat industry is not a realistic option, since it involves unsustainable costs and risks for the environment. In addition,

animal by-products constitute raw materials of interest for the pharmaceutical, food and leather industries.

In order to regulate the use and treatment of by-products, ensure their safety and avoid situations of risk of pathogens transmission (as it has happened in the past in cases such as the transmissible spongiform encephalopathies (TSE), foot-and-mouth disease or the presence of dioxins in feed), there are different European regulations that regulate the sanitary norms of animal by-products.

The regulation (EC) 56/2013, which amended the Annex IV to Regulation (EC) No 999/2001, establishes the normative for the use of animal proteins in animal nutrition to minimize the risk of TSE. Specifically, and with the exception of milk proteins, this law prohibits the use of proteins of bovine origin for animal nutrition. Thus, in Europe, plasma products for use in animal nutrition must be exclusively of porcine origin, decision derived from the opinion of the Scientific Steering Committee (SSC) in 27-28 November 2000, on the scientific basis to prohibit the animal proteins in the feed of all farmed animals, the SSC stated that there is no scientific evidence of TSE transmission in non-ruminant farmed animals. Therefore, it is established that blood products and hydrolyzed proteins derived from non-ruminants can be used in animal nutrition.

Regulation EC No 1069/2009 laying down health rules as regards animal by-products and derived products not intended for human consumption established that only animal by-products of category 3 (low risk) can be used to feed animals. Regulation 142/2011 implementing the above regulation establishes in its Annex I the definition of "blood product" as products derived from blood or fractions of blood, excluding blood meal; they include dried/frozen/liquid plasma, dried whole blood,

dried/frozen/liquid RBC or fractions thereof and mixtures. This annex clearly differentiates between blood products and blood meal, being the latter one defined as processed animal protein derived from the heat treatment of blood or fractions of blood. Therefore, according to the EU regulation 56/2013, the ban on the use of processed animal protein in the feed of farmed animals not applies to non-ruminant blood products as has not been considered as blood meal.

SDP, as a blood product, has to been obtained from only blood referred to in Article 10(a) and Article 10(b)(i) of Regulation (EC) No 1069/2009. This means that, for the production of blood products (including SDP), the source of raw material can come from either: 1) animals slaughtered which are fit for human consumption in accordance with Community legislation, but are not intended for human consumption for commercial reasons (10[a]), or 2) from animals that have been slaughtered in a slaughterhouse and were considered fit for slaughter for human consumption following an ante-mortem inspection (10[b][i]) .

In addition, blood products should follow the manufacturing requirements indicated in Section 2 of Chapter II of Annex X in Regulation (EC) No 142/2011:
Blood products must have been subjected to any processing method approved by the competent authority where it has been demonstrated to that authority that the final product has been sampled on a daily basis over a period of one month in compliance with the following microbiological standards:

- In the samples directly taken after heat treatment *Clostridium perfringens* must be absent in 1g of product.

- Samples taken during or after storage in the processing plant must have absence of *Salmonella spp.* in 25 g of product and less than 300 cfu/g of *Enterobacteriaceae* in 5 different samples taken from the same production batch.

Furthermore, all data analysis of the critical control points related with these microbiological standards including: the particle size, critical temperature and, as appropriate, the absolute time, pressure profile and raw material feed-rate, must be recorded and maintained to monitor the operation of the processing plant and must be made available to the Commission on request (Annex IV, Chapter III, processing methods, method 7).

The HACCP (Hazard Analysis Critical Control Points) System, which has scientific foundations and systematic character, allows identifying specific hazards and measures for their control in order to ensure the safety of food. It is an instrument to assess hazards and establish control systems that focus on prevention instead of the analysis of the final product (Food and Agriculture Organization of the United Nations (FAO), 2003). So, with an emphasis on prevention, one of the critical control points in plasma production is the collection of blood and the possible contaminating pathogens it may contain, especially, the presence of viruses from animals with subclinical infections and undetected viremia.

In the aforementioned European regulations, there is no explicit mention or reference about viruses and if their presence in the final product is permissible or not. This is considered a problem, especially, when the emergence or re-emergence of viral diseases appear and the blood by-products may be suspicious of transmitting the disease,

as it happened with the emergence of PEDV in North America (Pasick *et al.*, 2014).

In order to prevent these situations in the future and provide a greater biosafety product, other recommendations from important official organizations are available, such as the World Health Organization guidelines on viral inactivation and removal procedures intended to assure the viral safety of human blood plasma products (WHO, 2004) and the European Agency for the Evaluation of Medicinal Products (EMEA) note for guidance on viral validation studies (EMEA, 1996, 2001, 2006).

According to the WHO (2004) guidelines, inactivation steps should be able to remove or inactivate a wide range of pathogens. These guidelines consider that a robust, effective and reliable process should be able to remove or inactivate typically 4 log_{10} or higher amount of a given pathogen load. It is assumed that a production process that includes two robust steps able to remove or inactivate enveloped viruses is likely to result in a safe product. Also, a process that includes one robust step effective against non-enveloped viruses may result in a safe product, since these viruses are more difficult to remove or inactivate than enveloped ones. WHO guidelines are also in agreement with EMEA guidelines (EMEA, 1996, 2001) for virus validation studies for human plasma, which define a unit operation biosafety step under three categories (effective, ineffective or moderately effective). An effective step provides a reduction factor of at least 4 log_{10} and are unaffected by small perturbations in the different variables of the process. An ineffective step provides a reduction factor of 1 log_{10} or less, and a moderately effective step falls between the other two categories.

1.6 Pathogens of interest in the swine industry

Pathogen contamination of animal-based ingredients is a major safety concern for food and feed industries and for the global swine industry.

Pathogen presence in spray-dried porcine plasma (SDPP) may come from the raw material and/or by contamination during the manufacturing process. Therefore, preventive and control measures of the HACCP system and other regulations must be carried out.

Bacteria and viruses are the main pollutants in raw animal plasma. Both have different biological behavior. Bacteria, as unicellular organisms, can proliferate in raw plasma if the conditions of storage and temperature are not correct. However, viruses, as small infectious agents composed by genetic material (DNA or RNA) surrounded by a protein capsid and, in some genera, a lipidic envelope coming from cell membranes, could not replicate in raw plasma because they need to infect live cells (MacLachlan and Dubovi, 2011; Yoon, 2012). Furthermore, differences in size, composition and genetic material make viruses to behave differently in the face of possible methods of removal or inactivation.

In the case of bacteria, the European legislation requires the total absence of *Salmonella spp.* in 25 g of product and less than 300 cfu/g of total *Enterobacteraceae* organisms in 5 samples of the same production batch. In addition, during the validation of the process, it is necessary to demonstrate that the product has total absence of *Clostridium perfringens* in 1 g of product during 30 consecutive days.

There are no specific requirements in the legislation regarding viruses. Taking into account that the animals are *ante-mortem* inspected at the slaughterhouse, only the blood from healthy animals and of those

animals with subclinical infections and low viremia that are not detected by the official veterinarians could enter into the plasma manufacturing process.

Selected viruses of importance in the swine industry are listed in Table 1. They are ranked according to their thermal resistance or resistance to solvents and its ability to produce viremia. It is important to note that the presence or absence of lipid envelope is an influential differential factor to classify viruses according to their susceptibility to chemical biocides, but it does not allow predicting their susceptibility to thermal inactivation (Daniel and Talbot, 1987; Sauerbrei and Wutzler, 2009; Tuladhar *et al.*, 2012; Nims and Plavsic, 2013). Furthermore, the effectiveness of the inactivation processes may vary between the different strains of the same virus (Farcet *et al.*, 2012; Nims and Plavsic, 2013); so, it is recommended to test the most resistant strain (EMEA, 1996).

Table 1. Most important swine pathogens OIE 2017 ranked according to their thermal resistance or resistance to solvents and its ability to produce viremia.

Virus	Family	Genus	Genome	Envelope	Size (nm)	Genome Size (Kb)*	Thermal resistance	Solvent resistance	Duration viremia	Association of viremia with clinical signs	References
PRV	*Herpesviridae*	*Varicellovirus*	ssDNA	Yes	150-180	143.46	+	+	++	No/Yes	Bøtner, 1991; Nauwynck and Pensaert, 1994; Mettenleiter *et al.*, 2012
PRRSV	*Arteriviridae*	*Arterivirus*	(+)ssRNA	Yes	50-65	15.43	+	+	+++++	No	Cuartero *et al.*, 2002; Zimmerman *et al.*, 2012; Islam *et al.*, 2013
PEDV	*Coronaviridae*	*Alphacoronavirus*	(+)ssRNA	Yes	95-190	28.03	+	+	+/-	Yes	Carvajal *et al.*, 1995; Saif *et al.*, 2012; Jung and Saif, 2015
TGEV	*Coronaviridae*	*Alphacoronavirus*	(+)ssRNA	Yes	60-160	29.36	+	+	+/-	Yes	Cox *et al.*, 1990; Saif *et al.*, 2012)
BVDV	*Flaviviridae*	*Pestivirus*	(+)ssRNA	Yes	25-120	12.57	++	+	+	No/Yes	Bøtner and Belsham, 2012; Kirkland *et al.*, 2012; Tao *et al.*, 2013
CSFV	*Flaviviridae*	*Pestivirus*	(+)ssRNA	Yes	25-120	12.3	+ +	+	++++	No/Yes	Laevens *et al.*, 1998; Weesendorp *et al.*, 2011; Bøtner and Belsham, 2012; Kirkland *et al.*, 2012
SIV	*Orthomyxoviridae*	*Influenza virus A*	(-)ssRNA	Yes	80-120	13.15	+	+	+/-	No/Yes	Bøtner and Belsham, 2012; Van Reeth *et al.*, 2012

Table 1 (continuation). Most important swine pathogens OIE 2017 ranked according to their thermal resistance or resistance to solvents and its ability to produce viremia.

Virus	Family	Genus	Genome	Envelope	Size (nm)	Genome Size (Kb)*	Thermal resistance	Solvent resistance	Duration viremia	Association of viremia with clinical signs	References
ASFV	*Asfarviridae*	*Asfivirus*	dsDNA	Yes	200	170.1	++	+	+++	Yes	Sánchez-Vizcaíno and Arias-Neira, 2012; Guinat *et al.*, 2014
SVA	*Picornaviridae*	*Senecavirus*	(+)ssRNA	No	30	7.31	++	++	+	Yes	Joshi *et al.*, 2016; Leedom Larson *et al.*, 2017; Singh *et al.*, 2017
PPV	*Parvoviridae*	*Parvovirus*	ssDNA	No	18-26	5.07	++++	++++	+	No	Paul *et al.*, 1980; Streck and Felipe, 2012
PCV-2	*Circoviridae*	*Circovirus*	ssDNA	No	17	1.77	++++	++++	+++++	No/Yes	Segalés *et al.*, 2012; López-Soria *et al.*, 2014
SVDV	*Picornaviridae*	*Enterovirus*	(+)ssRNA	No	22-30	7.39	+++	++++	+	No/Yes	Dekker, 2000; Reid *et al.*, 2004; Alexandersen *et al.*, 2012
FMDV	*Picornaviridae*	*Aphtovirus*	(+)ssRNA	No	22-30	8.3	+ +	+ +	+	No/Yes	Alexandersen *et al.*, 2012; Toka and Golde, 2013
RVA	*Reoviridae*	*Rotavirus*	dsRNA	No	75	18.56	+++	+	+/-	Yes	Chang *et al.*, 2012; Araud *et al.*, 2016
HEV	*Hepeviridae*	*Hepesvirus*	(+)ssRNA	No	32-34	7.18	+++	++++	++++	No	Seminati *et al.*, 2008; Meng *et al.*, 2012; Busby *et al.*, 2013

1.7 Viral clearance procedures

As discussed above, viruses can be present in the initial raw material. In spite of implementing biosecurity measures (such as slaughterhouse *ante-mortem* inspection) and pathogen monitoring, it must be taken into account that all these control steps have limitations: human error, level of detection of the screening methods and specificity of pathogen monitoring methods (they are not universal, but limited to detect only the selected viruses) (WHO, 2004). These limitations indicate that the screening methods are not sufficient to ensure the biosecurity of the product by themselves; therefore, it is necessary to implement specific steps of inactivation and/or elimination of viruses within the production process (EMEA, 1996).

These stages focused on the inactivation and/or elimination of viruses must be validated conveniently, and the degree of viral infectivity loss during the manufacturing process must be assessed. It should be kept in mind that validation studies involve only an approximation to the elimination and/or inactivation obtained in the actual manufacturing process (WHO, 2004) since viruses and strains used in the experiments of validation may differ from those found in real plasma (Farcet *et al.*, 2012; Nims and Plavsic, 2013; Nims and Zhou, 2016). In consequence, it is recommended to work with the most resistant strain documented for validation purposes (EMEA, 1996).

To carry out this type of validation tests, a laboratory or pilot scale experiment should be designed, in which the starting material is experimentally inoculated with a sufficient quantity of virus. This artificially infected material must be treated with the selected method or methods, in a process that should be as similar as possible to that used in the large-scale manufacturing process (International Conference on

Harmonization (ICH), 1999).

For each process to be validated, the method of inactivation or elimination must be known and well described. For inactivation methods, the amount of virus inoculated should be calculated by titrating the viral stock solution and the initial infected sample (positive control of infection). In addition, sufficient samples should be collected at different times and also be titrated to know the reduction factor obtained at each time and, thus, be able to obtain the inactivation curve for the corresponding virus. In the case of elimination processes, the positive infection control and the final sample will be titrated to know the reduction factor, as well as a mass balance.

1.8 Conditions to consider to validate viral inactivation procedures

Several aspects should be taken into account when designing the validation steps of viral inactivation procedures:

- Selection of viruses: First, those viruses that are known to be naturally present in the product to be treated should be selected. These viruses are listed as "relevant viruses" according to ICHQ5A (International Conference on Harmonization (ICH), 1999). In some cases, relevant viruses cannot be used for different reasons, since they may not be adapted to cell line culture or it is not possible to generate sufficient virus titers. In these cases, model viruses or surrogates are used, which are viruses related to the virus of interest, belonging to the same genus or family, and having physicochemical characteristics similar to the virus of interest. In addition, to demonstrate the robustness of the process of inactivation or elimination, all types of viruses should be used to cover a wide range of characteristics:

viruses with and without envelope, different types of genomes, and different genome sizes.

- Reduction of the viral titer obtained.
- Dilution ratio: In inactivation and/or elimination studies, the inoculum should be added in a 1:9 ratio with respect to the sample. The rationale behind is to avoid changing the physical-chemical characteristics of the sample.
- The selectivity of the process against viruses of different types must be evaluated.
- The robustness of the system must be evaluated in the face of changes in the conditions of the process.
- Assessment of inactivation ratio and shape of the inactivation curve: It must be taken into account that first-order kinetics are not usually obtained; therefore, samples must be obtained at different times during the inactivation process to ensure the correct construction of the inactivation curve (International Conference on Harmonization (ICH), 1999). In the elimination processes a mass balance will be drawn up.
- Limitations of viral infectivity tests: The ability to detect low concentrations of viral particles depends, for statistical reasons, on the sample size analyzed. Therefore, the largest possible volume of those samples indicating that the entire viral titer has been inactivated or eliminated must be analyzed.
- Neutralizing antibodies in the sample: The presence of neutralizing antibodies in the sample to be treated may suppose a limitation for removal and inactivation techniques, since it can modify the amount of free virus and, at the same time, it also limits the titration techniques by neutralizing the viral infectivity.

1.9 Viral removal and viral inactivation methods used in plasma

Many different techniques have been described in the literature to inactivate or remove viruses from human plasma products (Morgenthaler, 2001; Rezvan *et al.*, 2006). Attending to their ability to be used in a wide range of products and manufacturing processes, their robustness, the high degree of scientific knowledge and contrasted data that support their results, the WHO, 2004 guidelines recognize as well characterized methods of inactivation pasteurization, dry heat, vapour heat, solvent/detergent mixtures and low pH. Likewise, it categorizes as well-recognized methods of viral removal the use of precipitation, chromatography and nanofiltration. The specific characteristics of these inactivation and removal methods are summarized in table 2.

Table 2. Well recognized viral inactivation procedures.

Treatment	Development	Selectivity	Warnings	Pros	Cons
Pasteurization	Heating at 60ºC for 10-11 hours	• Inactivates enveloped viruses. • Inactivates non-enveloped viruses	• Does not inactivate parvovirus B19. • HBV is relatively thermostable.	• Simple process. • Simple equipment.	• Protein denaturation. • Needs to use stabilizers to protect thermolabile proteins. • Stabilizers may protect viruses. • It may be necessary to remove stabilizers.
Solvent/detergent	Tri (n-butyl) phosphate (TNBP) at 0.3% to 1% with a non-ionic detergent at 1% 6 hours at 24ºC for Tween80 or 4 hours at 24ºC for Triton X-100	• Enveloped viruses	• Not effective against non-enveloped viruses	• No protein denaturation • High protein recovery • Simple equipment	• Not active against non-enveloped viruses • Solvent/detergent mixture must be removed
Acid pH	pH 4, 30-37 ºC, sometimes pepsin, minimum 20 hours	• Enveloped viruses	• Limited efficacy against non-enveloped viruses	• Reduce aggregation and anticomplementary activity of Ig fractions.	• Process limited to Immunoglobulins obtention.

Table 2 (continuation). Well recognized viral inactivation procedures.

Treatment	Development	Selectivity	Warnings	Pros	Cons
Dry heat	Dry heat at 80 ºC for 72 hours after lyophilization or dry heat at 100 ° C for 30 minutes.	• Enveloped viruses. • Non- enveloped viruses.	• Some non-enveloped viruses are resistant (EMCV, Canine parvovirus and human parvovirus B19).	• Effective against a wide range of viruses. • Is a terminal inactivation step, can be applied after other treatments on the final container.	• High temperatures. • Results are dependent on the residual moisture of lyophilization. • Complex validation.
Vapour heat	Heating the freeze-dried product by steam at 60 °C for 10 hours and then 80 °C for 1 hour.	• Enveloped viruses. • Non- enveloped viruses.	• Does not inactivate parvovirus B19.	• Effective against HAV.	• Complex validation. • Complex implementation.
Unsaturated fatty acids	Caprylic acid at pH below 6.5	• Enveloped viruses.	• Not effective against non-enveloped viruses.	• Used as a stabilizer in albumin pasteurization. And precipitating agent in the production of immunoglobulin G (IgG).	• Strict control. • At certain pH causes protein precipitation.

Table 2 (continuation). Well recognized viral removal procedures

Treatment	Development	Selectivity	Warnings	Pros	Cons
Precipitation	Cold ethanol precipitation.	• Enveloped viruses • Can be effective against non-enveloped viruses, including HAV and parvovirus B19.	• Low removal capacity.	• Partially separates viruses from proteins. • Greater removal capacity if filtration adjuvants are used.	• At working temperature (-3 and -5ºC) to preserve protein integrity, ethanol does not have disinfectant action.
Chromatography	Separation of solutes dissolved in a mobile phase, moving with different speed through a stationary phase. Basically, exchange and affinity chromatographies are used.	• Enveloped viruses • Can be effective against non-enveloped viruses, including HAV and parvovirus B19.	• Viruses may remain in the resin.	• High purification.	• Highly dependent of pH, ionic force, type of resin. • Resins must be disinfected after each process. • As the resin ages it loses efficiency.
Nanofiltration	Passing a fluid through a semipermeable membrane (15 to 40 µm pore diameter) at a certain pressure. This produces a separation depending on size.	• Enveloped viruses • Can remove non-enveloped viruses, as HAV and parvovirus B19.	• Limitation to remove small non-enveloped viruses.	• Specific to eliminate viruses. • Does not produce protein denaturation. • High degree of protein recovery.	• Virus removal is dependent of the pore size of filter used. • Filters can be damaged.

1.10 Applicability of plasma removal and inactivation procedures on SDP

Many of the different techniques used to inactivate or remove viruses from human plasma products are not suitable for the treatment of SDP during its manufacturing process. For example, solvent/detergent mixtures have been effectively used to inactivate lipid enveloped viruses (Horowitz, *et al.*, 1985a) and this is a method currently applied to treat raw native plasma, without fractionation. However, this process requires significant volume of solvent/detergent mixtures that must be removed and disposed and therefore is not suitable for the big volumes used in the SDP process. In the same way, enveloped viruses can be inactivated by the addition of unsaturated fatty acids (Horowitz *et al.*, 1988), or caprylic acid in combination with low pH and temperature (Lundblad and Seng, 1991; Korneyeva *et al.*, 2002). While many of these methods are very effective for plasma fractions (purified immunoglobulins or albumin), these methods are not appropriate for the production of SDP since they cause plasma protein denaturalization. The same happens with inactivation methods based on product heating, as pasteurization; it can also result in too many denaturized proteins or their precipitation and/or coagulation, with the consequent loss of biological activity of certain proteins. An alternative to inactivation is removal of the virus from plasma fractions by nanofiltration (Burnouf and Radosevich, 2003). However, this is not a practical option for industrial production of SDP since the volume of product to be processed is very large and most proteins are retained in the retentate. Similarly, other removal procedures used in the human plasma industry, like precipitation or chromatography are impractical due to the large volumes used by SDP manufacturing

industry. Other methods to treat raw native plasma, as methylene blue, are not easy to be implemented in the SDP manufacturing industry, since usually are intended to treat small volume each time. Furthermore, again it is needed to filtrate the resultant product to remove the methylene blue and its by-products; something that is not easy to perform on a large scale and that would involve delays in the production chain.

Fortunately, there are new viral inactivation methods under development that can be suitable to apply in the SDP large scale production process, as the Ultraviolet-C light (UV-C) irradiation.

1.11 Ultraviolet-C light (UV-C)

Ultraviolet light is a fraction of the electromagnetic spectrum located between X-rays and visible light (Jagger, 1967), spanning from 100 to 400 nm. In turn, the UV spectrum is divided in four wavelength ranges (Meulemans, 1986):

- Vacuum UV between 100 and 200nm
- UV-C between 200-280 nm
- UV-B between 280 and 315 nm
- UV-A between 315 and 400 nm.

UV-C is also known as "ultraviolet germicidal irradiation" (UVGI), since it covers the spectrum region with germicidal action and is easily distinguishable from UV-A and UV-B radiation (Kowalski *et al.*, 2000). UV has a higher DNA and RNA absorption at 254 nm, very close to the pick of maximum absorbance of DNA and RNA (260 nm). Therefore, 254 nm is the wavelength selected for UV-C treatment, where the maximum germinal action is achieved (Koutchma, 2009).

Unsaturated biological compounds show an absorption spectrum at wavelengths between 200 and 280 nm (Jagger, 1967). This occurs because the double bonds present in unsaturated compounds have two pairs of electrons. When these molecules are exposed to UV light, UV photons can be absorbed by the electron, causing an energetic jump in the electron, which passes from a basal to an excited state. This excitation can be transmitted through the molecule. If this energy is transmitted to an electron of the double bond, it becomes unstable, causing conformational changes (Jagger, 1967). When the nucleic acids, DNA and RNA, become exposed to UV light, the electrons of the nitrogenated bases absorb the UV photons, causing their jump to the excited state, what promotes the formation of covalent bounds between adjacent bases (Jagger, 1967). This process originates the cyclobutane pyrimidine dimers: thymine-thymine and thymine-cytosine dimers in DNA (Jagger, 1967) and thymine-uracil dimers in RNA (Jagger, 1967; Miller and Plagemann, 1974). The damage in the molecule is proportional to the amount of UV-C absorbed and, finally, it entails the inability to replicate and/or transcribe the genetic material, as well as the occurrence of mutations, which can lead to cell death (Snowball and Hornsey, 1988; Wellinger and Thoma, 1996; Sastry *et al.*, 2000). Furthermore, UV can induce cross-links between nucleotides and proteins in the capsid of viruses, originating damage in the viral capsid of DNA viruses (Miller and Plagemann, 1974; Kowalski, 2009).

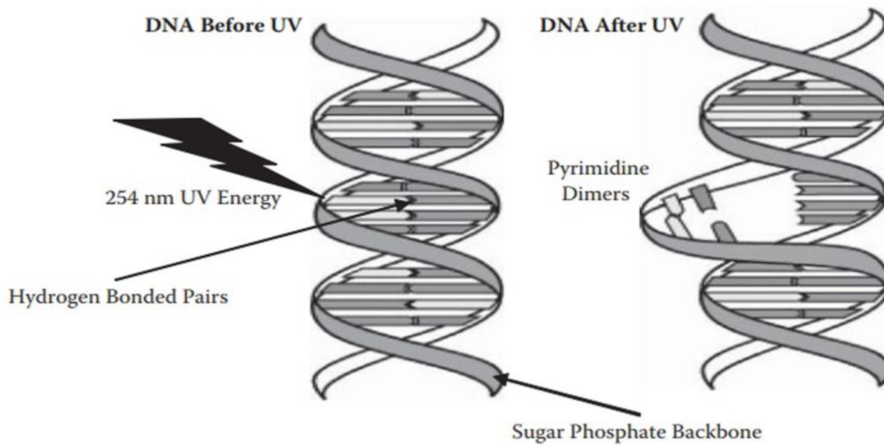


Figure 3. DNA structure before and after the UV-C photon absorption, which causes a pyrimidine dimer formation. From: Koutchma *et al.*, 2009.

Bacteria have mechanisms to repair the damage induced by UV (Atlas, 1995), while viruses can use the cell repair systems (Samad *et al.*, 1987). Photo-reactivation is a repair mechanism that restore the initial pyrimidine conformation, eliminating the pyrimidine dimer, by action of photolyase, an enzyme that needs blue or near UV light to catalyze the reaction (Sancar, 2004; Kowalski, 2009). Photolyase is present in bacteria, but not in viruses, being Fowlpoxvirus an exception (Srinivasan *et al.*, 2001). Another repair mechanism is called "dark repair", because does not need light to catalyze the reaction. In the "dark repair" scenario, the nucleotide is cleaved by the action of several enzymes. That process is less efficient tan photoreactivation and usually does not repair completely the induced damage (Kowalski, 2009). Interestingly, if

medium pressure lamps are used to generate de UV, enzymes as photolyase can be inactivated (Kalisvaart, 2004; Quek and Hu, 2008).

1.11.1 Generation of the UV-C and operation

Generation of UV irradiation is achieved by the use of mercury lamps. The classical mercury arc lamp consists of a hermetic tub of vitreous silica or quartz with two tungsten and alkaline metal electrodes at the ends, containing mercury and an inert gas, as argon (Phillips, 1983). When a high voltage is applied and passes through the electrodes, the discharge accelerate to high kinetic energies free electrons and ions in the mercury gas and the UV light is emitted when the mercury returns to the basal state (Koutchma, 2009). The most used mercury lamps are low and medium pressure ones (Koutchma, 2009).

The lamps are usually installed in a reactor in which the lamp or lamps are separated from the liquid by a quartz sleeve. The control system collects the most important technical characteristics associated with the lamps: temperature, electrical input (W/cm), UV output (W/cm), conversion efficiency, arc length and lamp lifetime.

There are many types of UV-C reactors. Basically, they can be divided into static reactors, in which the sample is irradiated without circulation, or continuous flow reactors, in which the sample is pumped to the sector where the ultraviolet lamps are located (Koutchma *et al.*, 2009).

The classical design of the static reactor is the bench-scale collimated beam (CB) device. It has been used as a standard method to measure the microbial dose response to UV-C and then, be able to extrapolate the data and scale up to UV-C systems that operate on a large

scale. The CB device consists of two low pressure mercury lamps suspended horizontally. UV-C light enters in the collimated tub and as a result, UV-C is collimated and irradiates the sample disposed in a petri dish (Qualls *et al.*, 1983). Large scale models based on CB (open-channel systems or closed-channel systems) are used in wastewater and drinking water disinfection systems (Wright and Cairns, 1998). These kinds of systems are useful to treat fluids that permit UV-C transmission easily. But the treatment of low UV-C transmission fluids requires modifications of the hydrodynamic conditions to achieve effective UV-C doses (Alberini *et al.*, 2015). Therefore, continuous flow reactors (laminar and turbulent continuous flow reactors) are recommended.

In laminar continuous flow reactors, the fluid circulates in a laminar regime, with a Reynolds number below 2500. The Reynolds number is a dimensionless parameter used in fluid mechanics that allows characterizing the movement of a fluid, determining if the flow is laminar or turbulent. The laminar regime implies that when the fluid is in motion, fluid flows in parallel layers, without disruption between layers. Very small irradiation chambers are used (between of 0.9-1.6 mm) in these reactors, improving the transmission of UV-C; the fluid is forced to pass through the irradiation chamber forming a very thin film, to avoid the superposition of fluid layers (Koutchma *et al.*, 2007).

Turbulent continuous flow reactors use a different strategy to improve the penetrability of UV-C into the material to be treated. By submitting the fluid to a higher flow, they manage to exceed the barrier of the 2500 Reynolds, so the system operates in a turbulent regime, meaning that the movement of the fluid particles is chaotic. In addition, the reactor uses coiled tubes to generate greater turbulence. In this way, a greater refreshment is achieved in the irradiation chamber and a greater

homogeneity of the irradiated sample (Koutchma, 2008; Alberini *et al.*, 2015). Due to the chaotic movement of the fluid particles, all of them can be close to the irradiation source and be irradiated with a higher probability.

1.11.3 Uses of the UV-C

UV-C irradiation technology has been used to inactivate several viruses in human plasma products. The first attempts to treat plasma derivatives in the 1950s failed to inactivate *Hepatitis B virus* (HBV) in plasma (Murray *et al.*, 1955), probably due to the difficulty to transmit the UV-C deeply to the sample and the high titer of HBV in plasma (Hart *et al.*, 1993). In addition, to obtain significant titer reduction values, the product had to be irradiated at such high doses that they ended up involving protein damage. Due to these technical problems, UV-C treatments were temporarily abandoned.

Fortunately, as UV-C technology developed, new strategies to irradiate high viscous and concentrated fluids were able to achieve good inactivation rates for different viruses, at the same time that the irradiation conditions allowed to maintain the protein integrity. Table 3 summarizes viral inactivation rates demonstrated by different authors.

Table 3. Viral inactivation rates obtained irradiating different plasma fractions or viral solutions.

PRODUCT	MICROORGANISM	UV-C DOSE	REDUCTION FACTOR (LOG)	REFERENCE
Viral solution	Human Immunodeficiency Virus	500 mJ/cm^2	2 $\log_{10}$	Nakashima *et al.*, 1986
Intravenous Immunoglobulin (IVIG)	T4 phage	510 mJ/cm^2	$\geq$ 3.3 $\log_{10}$	Hart *et al.*, 1993
IVIG	Semliki Forest Virus	510 mJ/cm^2	3.4 $\log_{10}$	Hart *et al.*, 1993
Albumin	Polio 2	510 mJ/cm^2	$\geq$ 6.4 $\log_{10}$	Hart *et al.*, 1993
Albumin	Herpes Simplex Virus	510 mJ/cm^2	$\geq$ 5.4 $\log_{10}$	Hart *et al.*, 1993
Albumin	Vaccinia virus	510 mJ/cm^2	$\geq$ 6.6 $\log_{10}$	Hart *et al.*, 1993
Human α_1-proteinase inhibitor (Prolastin®)	Porcine parvovirus	9 mJ/cm^2	4 $\log_{10}$	Wang *et al.*, 2004
Human α_1-proteinase inhibitor (Prolastin®)	Hepatitis AVirus	18 mJ/cm^2	4 $\log_{10}$	Wang *et al.*, 2004
Human α_1-proteinase inhibitor (Prolastin®)	Sindbis virus	40 mJ/cm^2	4 $\log_{10}$	Wang *et al.*, 2004
Human α_1-proteinase inhibitor (Prolastin®)	Reovirus type 3	74 mJ/cm^2	4 $\log_{10}$	Wang *et al.*, 2004
Human α_1-proteinase inhibitor (Prolastin®)	Adenovirus type 5	216 mJ/cm^2	4 $\log_{10}$	Wang *et al.*, 2004
Human α_1-proteinase inhibitor (Prolastin®)	SV 40	7 mJ/cm^2	4 $\log_{10}$	Wang *et al.*, 2004
Viral solution	SARS-CoV	867 mJ/cm^2	4.5 $\log_{10}$	Darnell *et al.*, 2004
FVIII, albumin, immunoglobulin, and fibrinogen	Murine Parvovirus	20 mJ/cm^2	>7 $\log_{10}$	Caillet-Fauquet *et al.*, 2004
FVIII, albumin, immunoglobulin, and fibrinogen	Encephalomyocarditis Virus	20 mJ/cm^2	5 $\log_{10}$	Caillet-Fauquet *et al.*, 2004
FVIII, albumin, immunoglobulin, and fibrinogen	Bovine Herpes virus type 1	24 mJ/cm^2	<3 $\log_{10}$	Caillet-Fauquet *et al.*, 2004
Prothrombin complex	Hepatitis A Virus	270 mJ/cm^2	$\geq$ 4 $\log_{10}$	Caillet-Fauquet *et al.*, 2004

Table 3 (continuation). Viral inactivation rates obtained irradiating different plasma fractions or viral solutions.

PRODUCT	MICROORGANISM	UV-C DOSE	REDUCTION FACTOR (LOG)	REFERENCE
Prothrombin complex	Canine Parvovirus	270 mJ/cm^2	$\geq$ 4 log_{10}	Caillet-Fauquet *et al.*, 2004
FVIII concentrate	Hepatitis A Virus	270 mJ/cm^2	5.5 log_{10}	Caillet-Fauquet *et al.*, 2004
FVIII concentrate	Bovine Parvovirus	270 mJ/cm^2	$\geq$ 4.6 log_{10}	Caillet-Fauquet *et al.*, 2004
FVIII concentrate	Pseudorabies Virus	270 mJ/cm^2	$\geq$ 5.5 log_{10}	Caillet-Fauquet *et al.*, 2004
FVIII concentrate	Bovine Viral Diarrhea Virus	270 mJ/cm^2	5.5 log_{10}	Caillet-Fauquet *et al.*, 2004
FVIII concentrate	Sindbis Virus	270 mJ/cm^2	5.5 log_{10}	Caillet-Fauquet *et al.*, 2004
FVIII concentrate	Parvovirus B19	1000 mJ/cm^2	$\geq$ 3.9 log_{10}	Sugawara *et al.*, 2001
Cell culture medium	Classical Swine Fever Virus	10000 mJ/cm^2	6.4 log_{10}	Freitas *et al.*, 2003
PBS	Bovine Viral Diarrhea Virus	1600 mJ/cm^2	4.3 log_{10}	Azar Daryany *et al.*, 2009
PBS + 5% Fetal Bovine Serum	Bovine Viral Diarrhea Virus	3200 mJ/cm^2	4.3 log_{10}	Azar Daryany *et al.*, 2009
PBS	Bovine Viral Diarrhea Virus	530 mJ/cm^2	1.75 log_{9}	Azar Daryany *et al.*, 2009
PBS + 5% Fetal Bovine Serum	Bovine Viral Diarrhea Virus	530 mJ/cm^2	0.33 log_{10}	Azar Daryany *et al.*, 2009
100% fetal Bovine Serum	Bovine Viral Diarrhea Virus	530 mJ/cm^2	No reduction	Azar Daryany *et al.*, 2009
Cell culture medium	Swine Influenza Virus	250 mJ/cm^2	~ 3.3 log_{10}	Cutler *et al.*, 2011
Cell culture medium	Porcine Reproductive and Respiratory Syndrome Virus	100 mJ/cm^2	~ 2.5 log_{10}	Cutler *et al.*, 2011
Cell culture medium	Bovine Viral Diarrhea Virus	300 mJ/cm^2	~ 5.8 log_{10}	Cutler *et al.*, 2011
Cell culture medium	Reovirus strain T3D[c]	300 mJ/cm^2	~ 1.7 log_{10}	Cutler *et al.*, 2011

UV-C is widely used for water disinfection (ÖNORM, 2001, 2003; Pirnie *et al.*, 2006). It was used for the first time in 1910 (Henry *et al.*, 1910), following the discovery of the first ultraviolet lamps. However, despite its effectiveness, its use was relegated in favor of chlorination, because it had a lower cost and did not need large equipment to be carried out. Over time, it was discovered that chlorination of water gives rise to dangerous oxidizing by-products. In addition, some human pathogens such as *Giardia* and *Cryptosporidium* resist chlorination.

The fact that the use of UV-C for water treatment does not generate by-products and that it is effective against the inactivation of *Criptosporidium parvumm* (Clancy *et al.*, 1998) and *Giardia* (Craik *et al.*, 2000), relaunched its use for water disinfection in the nineties.

There are a lot of references reporting good inactivation data against the main pathogens found in water (Hijnen, 2006), as viruses, such as Rotavirus, Adenovirus and Hepatitis A; bacteria, basically enterobacteria; but its major milestones are the inactivation of pathogenic protozoa of the genus *Cryptosporidium* and *Giardia*, as well as the inactivation of bacterial spores, such as those of *Clostridium perfringens.*

On the other hand, UV-C has been widely used in several food manufacturing industries as a safe inactivation technology to treat solid and liquid. Usually, UV-C is used in these industries as an alternative to thermal processing, due to UV-C allows to treat the product without altering its components and organoleptic characteristics (Guerrero-Beltran and Barbosa-Cánovas, 2004). The ease of use of the UV-C systems, its applicability for the treatment of large volumes of liquid, in addition to its continuous operation, that allows to carry out the

inactivation step "in process", without having to divert the product from the processing chain or stop the flow of this, together with the fact that it does not need the addition of additives to achieve the reaction and it does not generate by-products, make UV-C technology a great candidate for application as a biosecurity step in the processing of numerous food products. In this way, UV-C technology has been successfully used to treat juices (Sizer and Balasubramaniam, 1999; Bintsis *et al.*, 2000; Guerrero-Beltran and Barbosa-Cánovas, 2004; Koutchma *et al.*, 2007; Keyser *et al.*, 2008; Fredericks *et al.*, 2011; Groenewald *et al.*, 2013; Gayán *et al.*, 2014), tea (Monyethabeng and Krügel, 2016), milk (Matak *et al.*, 2005; Donaghy *et al.*, 2009; Christen *et al.*, 2013; Alberini *et al.*, 2015; Crook *et al.*, 2015), cheese (Ha *et al.*, 2016), wine (Fredericks *et al.*, 2011), egg (Unluturk *et al.*, 2010), dried seafood (Lee *et al.*, 2015) and sliced vegetables (Adhikari *et al.*, 2015; Gabriel, 2015; Martínez-Hernández *et al.*, 2015; Tarek *et al.*, 2016).

1.11.4 Inactivation kinetics

Traditionally, the inactivation kinetics by UV light has been described as a first-order reaction, in which it is assumed that the amount of reagent used (in this case, UV-C photons, λ= 254 nm) will be equal to the amount of product generated (cyclobutil pyrimidine dimers). This is based mainly in the First Law of Photochemistry (Grotthus-Draper Law): light must be absorbed by the molecule before any photochemical reaction can occur (Kowalski, 2009). This concept is completed by the Second Law of Photochemistry (Stark-Einstein Law), which postulates that if a photon is absorbed, it does not always have to lead to a reaction,

but if the reaction occurs, then only one photon is necessary for the formation of the photoproduct (Kowalski, 2009). Hence, the concept of quantum yield, because not all photons (energy quantums) are absorbed by the molecule.

1. Quantum yield: $\phi = \frac{Nc}{Np}$ where:

   Nc = number of molecules reacting chemically.

   Np = number of absorbed photons.

Due to the practical difficulty of these calculations, the Chick's law has usually been assumed. Chick's Law was developed in 1908 to describe the inactivation kinetics of bacteria in contact with disinfectants (Chick, 1908):

2. Chick's Law: $N = N_0 \mathrm{e}^{-kIt}$ where:

   N_0 = initial concentration of microorganisms before UV treatment.

   N = number of microorganisms remaining after UV treatment.

   I = UV intensity.

   t = exposure time.

   k = microorganism inactivation rate constant.

Usually, the inactivation constant (k) has been used to assess the susceptibility of a microorganism to de UV. This can be a good indicator as long as the inactivation kinetics of the microorganism will be linear; in this case, the kinetics will present only one inactivation constant (k). The problem begins when shoulders and tails appear in the inactivation curve, leading to different inactivation constants. Hiatt

(1964) introduced the concept of the two-stage model, as opposed to the first order equation, when trying to explain the appearance of shoulders, tails and kinetics of biphasic type. In the shoulder, no decrease in the population of the microorganism is observed despite increasing the dose of irradiation. In the tails, from a certain dose, there is a slowdown in the rate of inactivation of the microorganism, so that the population hardly decreases despite increasing the dose of irradiation (Hiatt, 1964). In all these situations it can be assumed that a fraction of the microorganism population is more susceptible to treatment, presenting a faster inactivation constant, while another fraction is more resistant, with a lower inactivation constant:

3. Biphasic equation (Cerf, 1977)

$$log10(N) = log10(N0) + log10\left(f * e^{-k_{max1}t} + (1-f) * e^{-k_{max2}t} \right)$$

Where:

$N0$ = initial bacterial concentration;

t = time;

f = fraction of the initial population in a major subpopulation,

$(1\text{-}f)$ = fraction of the initial population in a minor subpopulation,

k_{max1} and k_{max2} are the specific inactivation rates of the two populations, respectively.

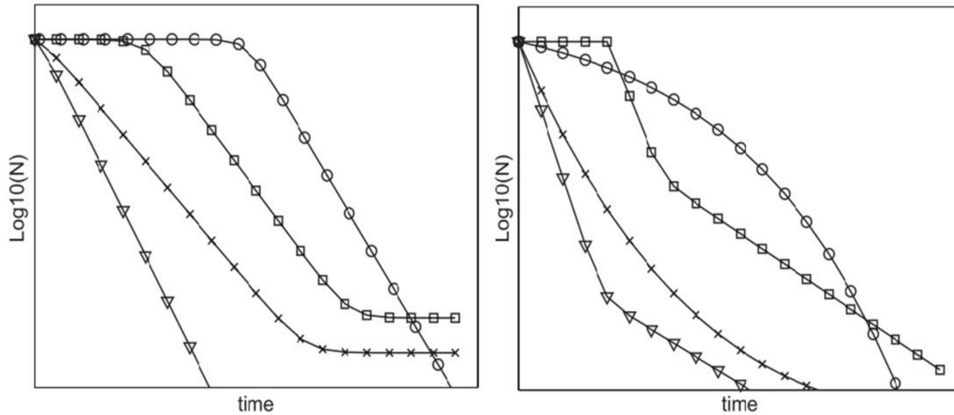
When the validation of an inactivation step is carried out, it is usual to report values such as the decimal reduction time, called D value, which is the time necessary to reduce the population of microorganisms in a logarithmic unit and the *z* value, which is the increment of energy (for example heat, UV, etc.) necessary for a 10-fold decrease in D value,

because the D value is dependent on the energy applied (Geeraerd *et al.*, 2000).

Calculating these values is important because it allows characterizing the system and the response of a given microorganism against the method of inactivation that is being applied. In addition, they allow to estimate when "X" logarithms of the microorganism in question will be inactivated, which allows extrapolation of the data to be scaled up to a higher scale.

In inactivation processes in which biphasic type kinetics are obtained and/or the appearance of tails and/or shoulders occurs, the calculation of these parameters can be complicated since their inactivation curves are not linear but are adapted to complex mathematic models. The complexity of the calculation of these parameters led many professionals to linearize the survival curves obtained in the inactivation experiments or only to work with the linear part of these (Hijnen, 2006). When this is done to calculate D values higher than 1 (for example the 4 D value) in biphasic or tail and/or shoulder kinetics, the estimation of this parameter is unsuccessful, since, due to takes into account only one inactivation constant (k), the calculation tends to lower D values, overestimating the inactivation capacity of the system.

Geeraerd et al. (2000, 2005), examining the literature related to thermal inactivation processes, were aware of this fact and developed a free software, GInaFiT, capable of performing advanced non-linear regression analysis applied to the kinetics of microbial inactivation. The program is able to interpret nine models of microbial inactivation kinetics, covering all known survivor curve shapes for vegetative bacterial cells (Geeraerd *et al.*, 2000, 2005).



Commonly observed types of inactivation curves. Left plot: linear (▽, shape I), linear with tailing (×, shape II), sigmoidal-like (□, shape III), linear with a preceding shoulder (○, shape IV). Right plot: biphasic (▽, shape V), concave (×, shape VI), biphasic with a shoulder (□, shape VII), and convex (○, shape VIII).

Figure 4: The eight most common types of inactivation curves. Only one of these curves is linear, describing the remaining seven inactivation kinetics that do not adapt to a log linear regression. From: Geeraerd *et al.*, 2005

GInaFiT software determines the goodness of fit in terms of root mean square error (RMSE) for all the tested models. The mathematical model that presents the lowest RMSE value is the model that fits better the data.

The equations that describe the different mathematical models are detailed below:

The Log linear model (Bigelow and Esty, 1920) presents the equation (1):

$$(1)\ log10(N) = log10\big(N(0)\big) - \left(\frac{k_{max}*t}{Ln(10)}\right)$$

Where *N* represents de microbial cell density, *N0* the initial microbial cell density, *t* is time; k_{max} is the first order inactivation constant and *Ln*(10) represents de decimal reduction time.

The log-linear plus tail model (Geeraerd *et al.*, 2000) uses the equation (2):

$$(2)\ logN = \log10\left(\left(10^{\log N0} - 10^{logN_{res}}\right)\right) - e^{(k_{max}\ t)} + 10^{logN_{res}}$$

Where *N* represents de microbial cell density, *N0* the initial microbial cell density, N_{res} is the number of resistant bacteria subpopulation, *t* is time and k_{max} is the inactivation rate of the log linear part of the curve.

The log-linear plus shoulder model (Geeraerd *et al.*, 2000, 2005) shows the equation (3):

$$(3)\qquad N = \frac{N0*e^{(-kmax*t)}*e^{(kmax*Sl}}{(1+\left(e^{(kmax*Sl)} - 1\right)*e^{(-kmax*t)}}$$

Where *N0* is the initial bacterial concentration; *t* is time; k_{max} is the inactivation rate and *S1* are the degrees of freedom.

Log-linear plus shoulder plus tail model (Geeraerd *et al.*,2000,2005) shows the equation (4):

$$(4)\ log10(N) =$$

$$= log10\left(\frac{((\ 10^{\log 10(\mathrm{N(0)})} - 10^{\log 10(\mathrm{Nres})}) * \mathrm{e}^{(-\mathrm{k_{max}}*\mathrm{t})} * \mathrm{e}^{(\mathrm{k_{max}}*\mathrm{Sl})}}{(1 + (\mathrm{e}^{(\mathrm{k_{max}}*\mathrm{Sl})} - 1) * \mathrm{e}^{(-\mathrm{k_{max}}*\mathrm{t})}}\right) + 10^{log10(Nres)}$$

Where *N0* is the initial bacterial concentration; N_{res} is the number of resistant bacteria subpopulation, *t* is time; k_{max} is the inactivation rate and *S1* are the degrees of freedom.

The biphasic model (Cerf, 1977) uses the equation (5):

$$(5)\ \ log10(N) = log10(N0) + log10\ (\ f * e^{-k_{max1}t}\ + (1 - f) * e^{-k_{max2}t}\ )$$

The Biphasic plus shoulder model (Geeraerd *et al.*, 2005) follows the equation (6):

$$(6)\ log10(N)$$

$$= log10\ (\mathrm{N0}) +\ \log 10 \left(\mathrm{f} * \exp(-\mathrm{kmax\ 1} * \mathrm{t}) * \frac{\exp(\mathrm{kmax1} * \mathrm{Sl})}{1 + \left(\exp(\mathrm{kmax1} * \mathrm{Sl}) - 1 * \exp(-\mathrm{kmax1} * \mathrm{t})\right) + 1(1 - \mathrm{f}) * \exp(-\mathrm{kmax2} * \mathrm{t}) * \frac{\exp(\mathrm{kmax1} * \mathrm{Sl})}{\left(1 + (\exp(\mathrm{kmax1} * \mathrm{Sl}) - 1) * \exp(-\mathrm{kmax1} * \mathrm{t})\right)^{\frac{\mathrm{kmax2}}{\mathrm{kmax1}}}}} \right)$$

Where *N0* is the initial bacterial concentration; *t* is time; k_{max1} and k_{max2} are the specific inactivation rates of the two populations and *S1* are the degrees of freedom used for the parameter estimation by GInaFiT

The Weibull model (Mafart *et al.*, 2002) uses the equation (7):

$$(7)\ \ log10(N) = log10\big(N(0)\big) - (\tfrac{t}{\delta})^{\ p}$$

Where *N* represents de microbial cell density, *N0* the initial microbial cell density, *t* is time, δ is a scale parameter denoted as the time for the first decimal reduction, and *p* is the shape parameter that describes concavity or convexity of the curve. If $p>1$ the curve shows convexity and if $p<1$ the curve is concave.

And the Weibull model plus tail (Albert and Mafart, 2005) follows the equation (8):

$$(8)\quad log10(N) = log10\ \left(\left(10^{\log N0} - 10^{logN_{res}}\right)\right) * 10^{(-\frac{t}{\delta})^{p}} + 10^{logN_{res}}$$

Where N_{res} is the number of resistant bacteria subpopulation, *N0* is the initial bacterial concentration and *N* represents de microbial cell density. δ is a scale parameter denoted as the time for the first decimal reduction, and *p* is the shape parameter that describes concavity or convexity of the curve. If $p>1$ the curve shows convexity and if $p<1$ the curve is concave.

Chapter 2

Hypothesis and objectives

SDP produced following current industrial manufacturing technology is considered a safe product. Its safety has been demonstrated in numerous *in vitro* and *in vivo* assays (Polo *et al.*, 2002, 2005, Opriessnig *et al.*, 2006, 2010, Pujols *et al.*, 2014, 2007, 2008, 2011; Shen *et al.*, 2011; Gerber *et al.*, 2014; Pujols and Segalés, 2014), being spray-drying process the main method of pathogen inactivation. However, since the plasma is a raw material and, despite it comes from healthy animals approved fit for slaughter for human consumption, the risk of pathogen transmission must be assessed throughout the whole manufacturing process. In consequence, in the interest of continuous biosafety improvement, new steps for the inactivation and/or effective removal of pathogens that would contribute to increase the overall biosecurity of the manufacturing process and the final product must be evaluated and duly validated.

From the swine industry point of view, special interest awakens from the application of redundant biosafety steps for the inactivation and/or removal of specific swine viruses. Moreover, there is a particular emphasis on non-enveloped ones, given their highly resistant nature and their ability to withstand thermal inactivation methods (Sofer and Lister, 2003; WHO, 2004; Nims and Plavsic, 2012; Nims and Zhou, 2016).

The spray-drying process bases its microorganism load reduction capabilities on the use of high temperatures and rapid drying of the sample (Straatsma *et al.*, 2007; Cal and Sollohub, 2010; Kuriakose and Anandharamakrishnan, 2010; Sollohub and Cal, 2010; Perdana *et al.*, 2013, 2015). The application of UV-C as a redundant biosafety step, based on an inactivation method that targets microorganism nucleic acids (Jagger, 1967), could be a good option to further reduce the bacterial and

viral loads of the product before being subjected to spray-drying. In consequence, UV-C irradiation would contribute increasing the global clearance capacity of the SDP production process, with low or no impact on the physicochemical characteristics of the plasma (Guerrero-Beltran and Barbosa-Cánovas, 2004). In addition, the UV-C step can be considered independent from the spray-drying process (WHO, 2004) and may be a synergistic process as both procedures have different inactivation targets.

Therefore, the general objective of this PhD Thesis was to evaluate the pathogen load reduction ability of an UV-C turbulent flow device, SurePure Turbulator™, on animal plasma experimentally spiked with different viruses and bacteria of importance for the swine industry.

Moreover, several specific objectives were designed within the framework of the general objective:

1. Validation of the SurePure Turbulator™ device for its use into the SDP production process:
   1.1. To determine the Log Reduction Factors (RF) and establish the survival curve of swine bacteria: *Salmonella typhimurium, Salmonella choleraesuis, Enterococcus faecium, Escherichia coli K88 and Escherichia coli K99*, artificially inoculated in commercially collected bovine plasma, and subjected to different UV-C irradiation doses.
   1.2. To analyze the Log Reduction Factors (RF) and define the survival curve of different enveloped* and non-enveloped** viruses artificially inoculated in

commercially collected bovine/porcine plasma, and subjected to different UV-C irradiation doses.

2. To carry out a pig bioassay to determine if UV-C irradiation of liquid commercially collected porcine plasma could inactivate viruses and bacteria that could be naturally present in porcine plasma.

* *Pseudorabies virus* (PRV), *Porcine reproductive and respiratory syndrome virus* (PRRSV), *Porcine epidemic diarrhea virus* (PEDV), *Bovine viral diarrhea virus* (BVDV), *Classical swine fever virus* (CSFV) and *Swine influenza virus* (SIV).

** *Porcine parvovirus* (PPV), *Swine vesicular disease virus* (SVDV), *Porcine circovirus 2* (PCV-2) and *Senecavirus A* (SVA).

Chapter 3

Ultraviolet (UV-C) inactivation of *Enterococcus faecium*, *Salmonella choleraesuis* and *Salmonella typhimurium* in porcine plasma

PLoS One.2017;12:e0175289

3.1 Introduction

Salmonella genus is a member of *Enterobacteriaceae* family. The genus *Salmonella* can be divided into two species (*S. enterica* and *S. bongori*) and *S. enterica* can be further subdivided into six subspecies. *Salmonella enterica var. enterica serovar typhimurium* is a cause of acute foodborne zoonosis worldwide (Hohmann, 2001) and pigs are important reservoirs (Gebreyes *et al.*, 2004). *Salmonella enterica var. enterica serovar choleraesuis* is frequently reported in North America and Asia (Gray *et al.*, 1995; Boyen *et al.*, 2008) causing disease in pigs, with a lower prevalence reported in Europe. *Enterococcus faecium* (*E. faecium*) NRRL B-2354 has been used as a model organism in thermal validation studies and is considered a suitable surrogate for foodborne pathogens to validate thermal processes used for dairy products, almonds, liquid foods and meat (Ma *et al.*, 2007; Kopit *et al.*, 2014).

The objective of this study was to assess bacterial inactivation efficiency of UV-C irradiation of liquid porcine plasma using a pilot plant system designed for irradiation of opaque liquids subjected to a turbid flow. The inactivation efficacy of the UV-C treatments was determined for *S. typhimurium* and *S. choleraesuis,* as well as *E. faecium.*

3.2 Material and methods

3.2.1 Bacterial strains and culture conditions

S. typhimurium (*ref UNI-UAB 46450)* and *S. choleraesuis (ref UMI-UAB 46429)* strains were provided by the UMI-UAB (Veterinary School,

Infectious Diseases Unit, *Universitat Autònoma de Barcelona*, Spain). They were cultured in several passes in tryptic soy agar (TSA) (Sigma-Aldrich) with increased amount of streptomycin from 0 to 500 µg/mL. Only colonies resistant to 500 µg streptomycin/mL of culture medium were used for the study. The antibiograms and the MIC showed a similar resistant profile for both *Salmonella* spp. They were resistant to ampicillin, ciprofloxacin, nalidixic acid, gentamicin, streptomycin, tetracycline, colistin, sulfamethoxazole, trimethoprim, chloramphenicol, kanamycin, ceftazidime, combination of trimetoprim and sulfamethoxazole, neomycin, rifampicin, tiamulin and tylosin.

Enterococcus faecium (strain NRRL B-2354, ATCC 8459) was grown in brain-heart infusion agar (BHIA) (Sigma-Aldrich).

3.2.2 Inoculum preparation

Salmonella spp. inocula for both strains were prepared in TSA media containing 500 µg steptomycin/mL. After 24 hours of growth at 37ºC, bacteria were harvested by a Kolle handle and resuspended in 10 mL PBS. The inoculum for *E. faecium* was prepared after growth in BHIA for 24 h at 37ºC.

Liquid fresh plasma from industrial abattoirs may contain different microorganisms. For that reason, it was decided to sterilize 2.5 kg of spray-dried porcine plasma (lAP820P, APC-Europe S.A., Granollers, Spain) by gamma-cobalt-60 irradiation at 10 KGray (Aragogamma S.A, Les Franqueses del Vallès, Barcelona, Spain) to eliminate any potential bacteria. The γ-irradiated SDP was diluted 1:11 in water (2.5 kg SDP + 25.0 kg of water) to obtain 27.5 kg of liquid

plasma at 9.66, 9.50 and 9.11% solids for *S. choleraesuis, S. typhimurium* and *E. faecium*, respectively. An inoculum of each individual bacterium containing 10^9 cfu/mL was prepared and used to infect 24 L aliquots of plasma to achieve a minimum final titer of approximately 10^6 cfu/mL. All bacterial handlings were done in a sterile laminar flow cabin to protect the staff and microbiological cultures.

3.2.3 UV-C irradiation

After mixing the bacteria inoculum with the 24 aliquot of plasma, the total volume was divided into three 8 L sub-aliquots. At time zero, a positive control 15 mL sample was collected 5 min. after bacteria were mixed with the plasma. Samples of each sub-aliquot were consecutively irradiated at 750, 1500, 3000, 6000 and 9000 J/L. During the UV-C treatment, sequential 15 mL samples were taken at each time-dose of irradiation. As a negative control, a 15 mL sample of liquid plasma was obtained before bacteria inoculation.

After UV-C irradiation, 1 mL samples were ten-fold diluted in peptone water and 0.1 mL of the dilatant was inoculated onto 25 mL TSA plates containing streptomycin (500µg/mL) to count *S. typhimurium*, or *S. choleraesuis* and 0.1mL of the dilatant was inoculated onto 25 mL BHIA plates for count of *E. faecium* colonies that survived the different UV-C irradiation doses.

Count results were expressed as a Log 10 CFU/mL, and survival curves were plotted as Log10/mL as function of UV-C dose (J/L).

3.2.4 Settings of pilot scale UV-C system

The UV-C reactor system SP1 produced by Sure Pure Operation AG (Zug, Switzerland) was used (see Annex 1 Fig.13).

Plasma was inoculated and recirculated many times through SP1 circuit to achieve the required UV-C dose versus time. Liquid flow was controlled by a flow meter. Flow rate was adjusted to 4000 L/h. The time spent by the liquid (8L total volume) to pass through the system once was 7.2 s, delivering 22.95 J/L or 22.94 mJ/cm^2 per cycle.

At the start of the process, the flow rate was stabilized for 5 min water recirculation. Water was replaced by plasma and then the treatment process was initiated. First, with the UV-C lamp turned off, a positive control (time 0) sample was collected allowing the product to recirculate throughout the SP1 system at 4000 L/h during 5 minutes. After this step, the UV-C lamp was switched on and irradiation was started. Samples were collected at defined times into sterile containers for microbiological analysis. Samples were collected at different UV-C doses (0, 750, 1500, 3000, 6000 and 9000 J/L) corresponding to different intervals of irradiation time (0, 4’31”, 7’49”, 15’35”, 31’05” and 46’28”).

3.2.5 Modeling of inactivation

Bacterial inactivation due to thermal and non-thermal processes can display one of eight possible curve shapes. Log linear inactivation modeling fails to accurately assess the majority of the survival curves (Geeraerd *et al.*, 2005).

In order to analyze the inactivation curves and to assess if bacteria inactivation was linear or non-linear, the GInaFiT software was used to obtain non-linear survival curves (Geeraerd *et al.*, 2000) and to test log-

linear plus tail (Geeraerd *et al.*, 2000), Weibull (Mafart *et al.*, 2002), Weibull plus tail (Albert and Mafart, 2005) and biphasic (Cerf, 1977) models. Inactivation modeling equations are found in the Chapter 1: Introduction (section 1.11.4 Inactivation kinetics).

3.2.6 Statistical analysis

Data were expressed by means of Log10 values and standard deviations of three independent experimental batches.

Mean, standard deviations, ANOVA and F-Test for comparisons were calculated with Excel 2007 (Microsoft Office) to determine significant differences between doses. Tukey test was calculated with Statgraphics Centurion XV version 15.2.14 (StatPoint Technologies Inc, Warrenton, Virginia) to determine significant differences between treatments for both Salmonella and *E. faecium*. Differences at $p<0.05$ were considered significant.

Mean square error (MSE), goodness of fit in terms of root mean square error (RMSE), correlation coefficient (R^2) and adjusted correlation coefficient (adj-R^2) values were calculated with GInaFiT software (Geeraerd *et al.*, 2005). To choose the inactivation model with the best fit, the model with the smallest RMSE was chosen (Geeraerd *et al.*, 2005).

3.3 Results

There were significant differences ($p<0.05$) between treatments for the three tested bacteria, except for the first treatment on *S. typhimurium* (750 J/L), which was not significantly different from time 0.

Plasma inoculated with *S. choleraesuis* had an initial count of 7.97 $\log_{10}$/mL and when UV-C treated showed a curve with a regression coefficient of R^2 = 0.9867 (Table 4). *S. choleraesuis* displayed a robust and replicable reduction rate of 5.5 $\log_{10}$ between 0 and 3000J/L of UV-C. However, a complete inactivation of *S. choleraesuis* (7.97 log) was achieved at 9000 J/L (Fig. 5)

Table 4. Statistical parameters of three models for inactivation of *S. choleraesuis*, *S. typhimurium* and *E. faecium*.

	S. choleraesuis		***S. typhimurium***		***E. faecium***	
	Biphasic	**Weibull**	**Biphasic**	**Weibull plus tail**	**Biphasic**	**Weibull**
MSE[1]	0.1495	0.5116	0.1367	0.0588	0.2577	0.5420
RMSE[2]	**0.3867**	0.7152	0.3698	**0.2425**	**0.5076**	0.7362
R-Square	0.9867	0.9511	0.9698	0.9870	0.9684	0.9288
R-Square adjusted	0.9838	0.9446	0.9622	0.9837	0.9617	0.9193
4D reduction is reached at (J/L)[3]	**2301**	2125	3364	**3186**	**3364**	3984

[1]MSE: Mean sum of squared error.

[2]RMSE: Root mean sum of squared error.

[3]4D reduction: UV irradiation in J/L at which achieved 4 Log_{10} reduction.

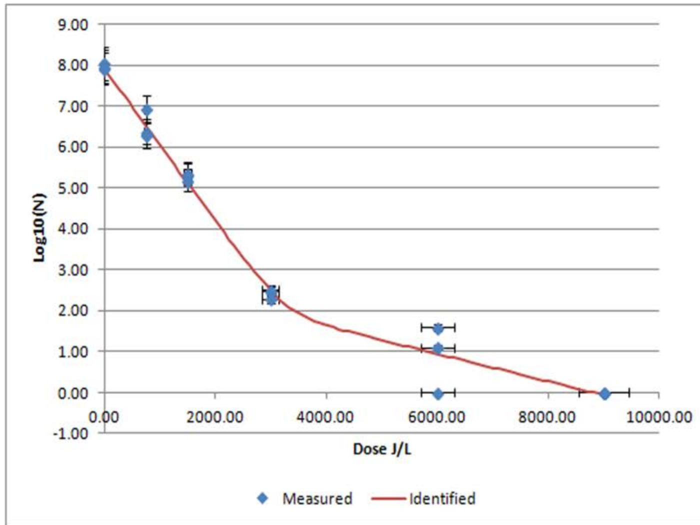


Fig. 5. Mean *S. choleraesuis* $\log_{10}$/mL values after UV-C irradiation of porcine plasma at different UV irradiation doses.

Blue diamonds indicated measured results of *S. choleraesuis* at different UV-C irradiation doses expressed as mean ± SEM (n=3 replicates). Red line is the identified curve according to the biphasic inactivation curve model.

Data for *S. choleraesuis* fitted slightly better with the biphasic model having the lowest RMSE (Table 4).

Plasma inoculated with *S. typhimurium* had an initial count of 6.85 ± 0.04 $\log_{10}$/mL and, after UV-C treatment, the decrease in bacterial counts showed a curve adjusted with the Weibull plus tail model, with a regression coefficient of $R^2 = 0.9870$ (Table 4). *S. typhimurium* displayed a robust and replicable inactivation of 3.59 $\log_{10}$ between 0 and 3000 J/L (Fig. 6). A residual of around 2 log of *S. typhimurium* counts was observed after irradiation at a dose of 9000 J/L.

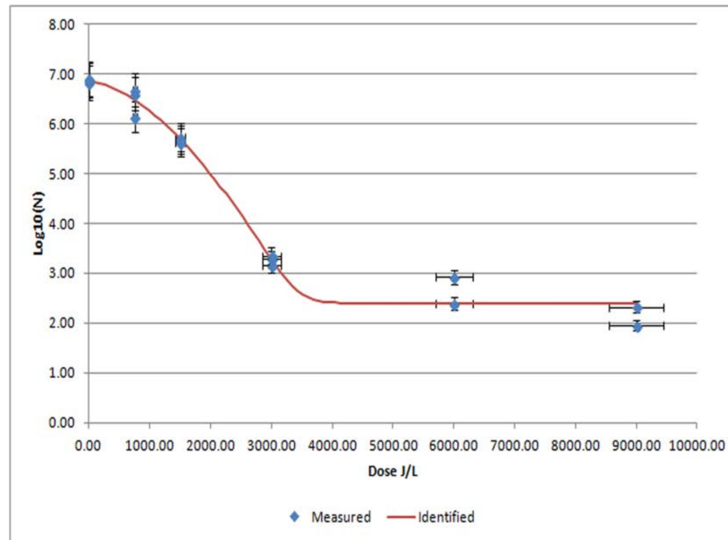


Fig. 6. Mean *S. typhimurium* log_{10}/mL values after UV-C irradiation of porcine plasma at different UV irradiation doses.

Blue diamonds indicate measured results of *S. typhimurium* at different UV-C irradiation doses expressed as mean ± SEM (n=3 replicates). Red line is the identified curve according to the Weibull plus tail inactivation curve model.

E. faecium was inoculated at 6.22 ± 0.13 log_{10}/mL in liquid plasma and after UV-C treated, a biphasic growth curve with a regression coefficient of R^2 = 0.9684 was observed (Table 4, Fig 7). At UV-C irradiation dose of 9000 J/L there was a total lack of detectable bacterial growth.

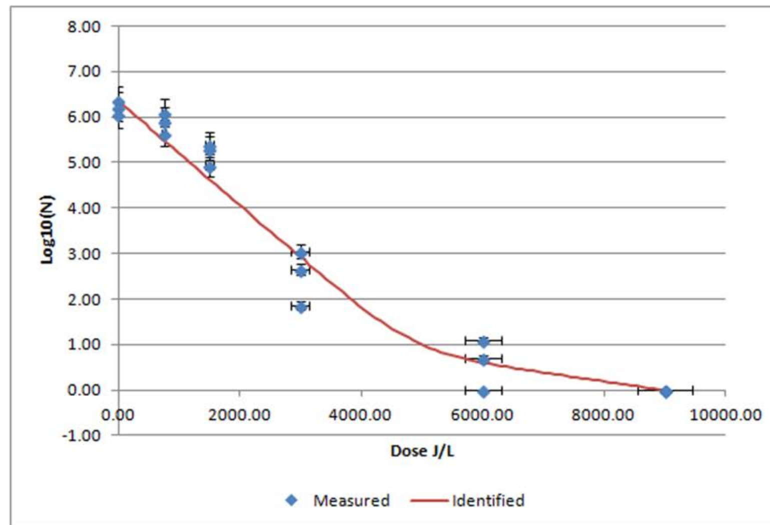


Fig. 7. Mean *E. faecium* $\log_{10}$/mL values after UV-C irradiation of porcine plasma at different UV irradiation doses.

Blue diamonds indicated measured results of *E. faecium* at different UV-C irradiation doses expressed as mean ± SEM (n=3 replicates). Red line is the identified curve according to the biphasic inactivation curve model.

3.4 Discussion

In a previous study using a similar pilot plant Sure Pure system (SP1) (Polo *et al.*, 2015), UV irradiation of liquid plasma at doses higher than 2295 J/L inactivated more than $10^{5.2}$ $TCID_{50}$/mL of PPV inoculated in the liquid plasma. Also, in the same publication UV-C irradiation of liquid plasma prior to spray-drying did not negatively affect the productive parameters of post-weaned pigs fed a diet containing UV-C treated SDPP. Although UV-C irradiation was effective for inactivation of PPV, a model for heat resistant viruses, it was also necessary to investigate the effect of UV-C irradiation on the survival of different

bacteria of interest such as *S. typhimurium, S. choleraesuis* and *E. faecium* when inoculated in liquid plasma. Bacteria have more mechanisms of genome reparation than viruses and it can be argued that the effect of UV-C irradiation may be less efficient in bacteria compared with simpler microorganisms as viruses. In addition, it was necessary to know if bacteria that are able to develop antibiotic resistance may be more difficult to eliminate when UV-C irradiation is applied. In the present experiments, a reduction of almost 4 log_{10} for each bacterium was determined when irradiation with UV-C doses around 3000J/L were used. According to calculations, a four decimal (4D) reduction was reached at 2301 J/L for *S. choleraesuis*, 3186 J/L for *S. typhimurium* and 3364 J/L for *E. faecium* when using the best-fit inactivation model curve.

E. faecium and *S. choleraesuis* had total inactivation by UV-C at 9000 J/ L with a biphasic curve in both cases. However*, S. typhimurium* displayed an inactivation resistance with a tail effect. The tail survival effect of *S. typhimurium* may be related to a bacteria resistant subpopulation. *S.typhimurium* showed a N*res* parameter (Number of resistant bacterial subpopulation) in a Weibull plus tail model of 2.40 log_{10} ±0.12. These results agree with data obtained by Luksiene et al. (Luksiene *et al.*, 2007), who found a similar distribution of inactivation results working with pulsed UV-light. Usually, microorganism inactivation by UV-C light follows a first order kinetics in liquids, but some agents can exhibit a sigmoidal shape with shoulder or tail curves (Baysal *et al.*, 2013). In the present work, the GINAFiT software (Geeraerd *et al.*, 2005) was used to test the best-fit model for the obtained data, using the goodness of fit in terms of RMSE to select the model for each individual bacterial inactivation curve. Both *Salmonella spp*. strains used in this study were resistant to streptomycin. Therefore, both strains

contained genes involved in antibiotic resistance, but UV-C inactivation curves for both strains were different; a higher UV-C resistance was found for *S. typhimurium* compared to *S. choleraesuis*. These results may suggest that the genes involved in antibiotic resistance may not preclude the resistance of these bacteria to UV irradiation and different gene resistance development or resistance mechanism may be involved.

E. faecium is a food-borne surrogate bacterium with no antibiotic resistance genes that is commonly used to compare its inactivation kinetics with those obtained with pathogenic bacteria in industrial manufacturing processes. In this study, the inactivation curve for *E. faecium* was similar to the inactivation curve for *S. choleraesuis*, although each bacterium had different best-fit inactivation curves. Therefore, it is suggested that *E. faecium* can be used as food surrogate for this bacteria in UV-C studies for animal plasma conducted at a commercial level. In contrast, the inactivation behavior of *E. faecium* was significantly different to the curve observed for *S. typhimurium,* suggesting that this food probiotic bacterium is not a good surrogate for *S. typhimurium*.

In conclusion, these results provide evidence of the 4 $\log_{10}$ reduction of studied microorganisms at affordable levels of UV-C irradiation. In addition, results suggested that UV-C technology can be used as an additional biosafety feature to minimize risk of biohazards that may be present in biological products like liquid plasma.

Chapter 4

Evaluation of ultraviolet-C and spray-drying processes as two independent inactivation steps on enterotoxigenic *Escherichia coli* K88 and K99 strains inoculated in fresh unconcentrated porcine plasma

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4.1 Introduction

UV exposure at a wavelength of 254 nm (UV-C) is a non-thermal process that has a germicidal effect by causing thymine-thymine and thymine-cytosine dimers in DNA and thymine-uracil dimers in RNA, which disrupts microbial reproduction (Jagger, 1967). During the spray-drying process (See Chapter 1: Introduction and Annex 2), thermal inactivation, high pressure and rapid dehydration are the phenomena involved in microbial inactivation. Although the most important site of damage caused by dehydration is the cytoplasmic membrane (Crowe *et al.*, 1987; Lievense and Van't Riet, 1994; Perdana *et al.*, 2013; Huang *et al.*, 2017), dehydration also produces damage to DNA/RNA and protein (Lievense *et al.*, 1992). Thus, the sequential action of both methods for the plasma production process is promising to inactivate microorganisms, since both damage different targets involved in microbial inactivation.

Enterotoxigenic *Escherichia coli* (*E. coli*) is one of the main causes of enteric disease and death in newborn and weaned pigs (Francis, 2002) and is the major cause of neonatal diarrhoea in calves (Acres, 1985). *E. coli* requires the expression of adhesion fimbriae (adhesins), which are encoded in plasmids, to be adhered to the intestinal epithelium. *E. coli* expressing K88 adhesin is mainly found in pigs (Gaastra and De Graaf, 1982), while K99 is the main adhesion antigen found in bovine species (Tzipori, 1981), although K99 can also be found in ovine and porcine species (Gaastra and De Graaf, 1982).

The aim of this study was to assess the effectiveness of a UV-C treatment system on *E. coli* inactivation after inoculation in fresh unconcentrated liquid porcine plasma. In addition, a second objective was to test the effectiveness of the spray-drying process on the

inactivation of *E. coli* at two different outlet temperatures, at the regular outlet temperature normally used by the industry (80ºC) and at lower outlet temperature (70ºC).

4.2 Materials and methods

4.2.1 Bacterial strains and test products

Two strains of *E. coli* were used in the present study: an isolate from swine expressing the K88 adhesin, and a second isolate from bovine expressing the K99 adhesin (both isolates were kindly provided by Dr. Antonio Juárez. University of Barcelona, Spain). A 0.3 mL volume of *E. coli* isolates were cultured in 100 mL of LB media (Sigma-Aldrich) at 37ºC and 150 rpm for 18 hours. The cells were subsequently concentrated by centrifuging (1000 x g for 20 minutes at 4ºC) using sterilized 40 mL tubes containing 20 mL of culture media. The remaining culture media was removed by resuspending the cell precipitate in 20 mL of sterile 0.01 g/mol phosphate buffer Saline (PBS). After resuspension, it was centrifuged again as described above and the resulting cell precipitate that was resuspended again in 500 mL of PBS reaching a final titer of 8.98 $\log_{10}$ CFU/mL for K88 and 8.91 $\log_{10}$ CFU/mL for K99.

Fresh liquid porcine plasma from the production plant of APC-Europe S.A., (Granollers, Spain) was used for these trials. This plasma was obtained by centrifugation of blood from pigs processed at a local officially inspected abattoir.

4.2.2 Settings of pilot scale UV-C system

The setting of pilot scale UV-C used is described in Annex I.

4.2.3 UV-C test

A total of 60 kg of plasma were used for the present study, 30 kg for each of the tested *E. coli* strains. For each isolate, the 30-kg batch was divided into three 10-kg sub-batches to conduct tests in triplicate. Because liquid fresh plasma from the abattoir may contain different microorganisms, the initial 60 kg of plasma product was treated by UV-C at 10,000 J/L for one hour to inactivate any potential bacteria prior to artificial inoculation with *E. coli*.

Plasma was spiked with an inoculum of 90 mL of *E. coli* K88 (ratio 1/330) and 220 mL in the case of *E. coli* K99 (ratio 1/138). After inoculation, the liquid was recirculated through the UV-C device for 3 minutes before activating the UV lamp. At time 0, a non-processed sample was taken and the UV lamp was activated. During the UV-C treatment, 150 mL samples were taken when doses reached 750, 1500, 3000, 6000 and 9000 J/L (equivalent to 4’47”, 9’51”, 18’54”, 37’34”, 56’00”).

After each UV-C irradiation dose, 1 mL samples were ten-fold diluted in peptone water and 0.1 mL inoculated by duplicates onto TBX agar plates (Sigma-Aldrich) and incubated for 24 hours at 37ºC. Plates with more than 20 and less than 300 colonies were counted and results expressed as $\log_{10}$/mL.

4.2.4 Spray-drying test

A total of 7 kg of fresh plasma from a commercial manufacturing plant was previously UV-C treated at 10,000 J/L prior to inoculation with the *E. coli* strains to eliminate any other bacteria present in the initial raw material. Half amount (3.5 kg) of this UV treated plasma was spiked with

the swine *E. coli* K88 isolate at a ratio of 1/47 reaching a final titer of 7.31 ± 0.39 log_{10}/mL and the other half with the bovine *E. coli* K99 isolate, at a ratio 1/18 reaching a final titer of 7.66 ± 0.11 log_{10}/mL. From each of the 3.5 Kg inoculated plasma aliquots, two bottles of 750 mL were obtained and spray-dried in a lab drier (Büchi Mini Spray Dryer B-290, Büchi Labortechnik, Switzerland) at two different conditions: inlet temperature at 220 ± 1ºC and outlet temperature at 80 ± 1ºC or 70 ± 1ºC, after stabilization of the spray-drier with water and non-inoculated control plasma. All studies were performed in triplicate. Air flow through the column was set at 20 - 27 m^3/h at 20ºC. Estimated dwell time was <1 second.

Once SDP was obtained at the two designated outlet temperatures, 3 tubes containing 0.5 g of dried plasma for each condition were obtained and the dry samples were re-suspended in water at a ratio of 1:9. From this re-suspension, 0.1 mL was seeded in TBX agar for 24 h at 37ºC. Colony counting was performed as indicated in the previous section. Results were expressed as a log_{10}/g of solids according to the equation: $log_{10}/g = log_{10}$ (CFU/mL) / [(% solid content of re-suspended sample)/100].

4.2.5 Modeling of inactivation

The GInaFiT software was used to calculate and plot non-linear *E. coli* survival curves. The log-linear plus tail (Geeraerd *et al.*, 2000) and Weibull plus tail (Albert and Mafart, 2005) models were tested. These equations are described in Chapter 1: Introduction, section 1.11.4 Inactivation kinetics.

4.2.6 Statistical analysis

Data were expressed by means of Log_{10} values and standard deviations of three independent experimental batches. Mean, standard deviations, ANOVA and F-Test for comparisons were calculated with Excel 2007 (Microsoft Office). The LSD (Least Significant Difference) test was calculated with Statgraphics Centurion XV version 15.2.14 (©StatPoint Technologies Inc, Warrenton, Virginia) to determine significant differences between treatments. Differences at $P<0.05$ were considered significant.

Mean square error (MSE), goodness of fit in terms of root mean square error (RMSE), correlation coefficient (R^2) and adjusted correlation coefficient (adj-R^2) values were calculated with the GInaFiT software (Geeraerd *et al.*, 2005). The smallest RMSE determined the inactivation model with the best fit (Geeraerd *et al.*, 2005).

4.3 Results

4.3.1 UV-C test

Plasma inoculated with *E. coli* K88 strain had an initial count of 6.46 ± 0.04 log_{10}/mL. After UV-C treatment at 3000 J/L, bacterial counts showed a significant reduction of 4.34 log, describing a curve adjusted to the log linear plus tail model (Fig. 8) with a regression coefficient of R^2=0.95 (Table 5). At doses of 6000 and 9000 J/L, residual *E. coli* populations of 1.18 ± 0.30 and 1.12 ± 0.30 log_{10}/mL were counted, respectively. The UV-C doses required to have 4 log_{10} reduction (log10R) was predicted to be 3105 J/L.

Plasma inoculated with the strain *E. coli* K99 had an initial count of 6.78 ± 0.67 log_{10}/mL. After UV-C treatment, bacterial counts decreased significantly, showing a curve adjusted with the Weibull plus tail model, with a regression coefficient of R^2=0.923 (Table 5). There was a 3.97 log_{10}/mL reduction of the initial count between 0 and 3000 J/L (Fig. 8). Residual populations of 2.30 ± 0.08 and 2.11 ± 0.15 log_{10}/mL were counted after irradiation at doses of 6000 and 9000 J/L, respectively. The 4 log10R was predicted to be achieved at 3427 J/L.

Table 5. Statistical parameters of the two models for inactivation applied to results obtained with strains *E. coli* K88 *and* K99.
*MSE: Mean sum of squared error; †RMSE: Root mean sum of squared error; ‡4D reduction: UV-C dose irradiation in J/L at which a 4 Log reduction was achieved.

	***E. coli* K88**		***E. coli* K99**	
	Log linear plus tail	**Weibull plus tail**	**Log linear plus tail**	**Weibull plus tail**
MSE*	0.2594	0.2686	0.3874	0.3835
RMSE†	**0.5093**	0.5182	0.6224	**0.6193**
R-Square	0.9504	0.9511	0.9167	0.9235
R-Square adjusted	0.9457	0.9438	0.9048	0.9058
4D reduction‡ reached at ($J\ L^{-1}$)	3105.9	3105.9	3427.2	3427.2

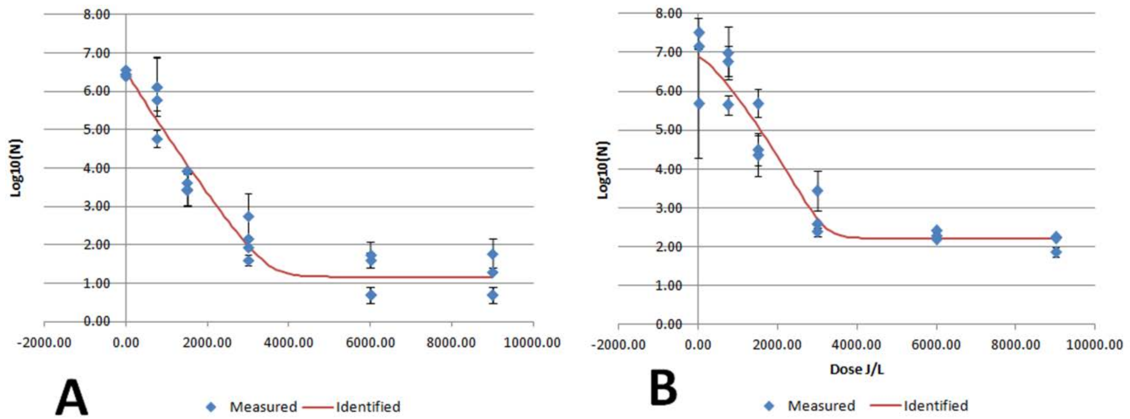

Fig. 8. Inactivation kinetics of the strains *E. coli* K88 (A) and *E. coli* K99 (B) after UV-C irradiation.

E. coli K88 presented a Log linear plus tail inactivation kinetics while *E. coli* K99 showed a Weibull plus tail inactivation kinetics. "Measured" indicate the real data obtained during the experiment. "Identified" is the best model fit for prediction kinetics obtained by the GInaFiT program.

4.3.2 Spray-drying test

Full inactivation of strains *E. coli* K88 and K99 was achieved in all spray-dried samples dehydrated at an inlet temperature of 220 ± 1ºC and both outlet temperatures of 80 ± 1ºC or 70 ± 1ºC (Table 6).

Table 6. Effect of the outlet temperature on the inactivation of each *E. coli* strain tested.

	***E. coli* K88 strain (CFU Log_{10} g^{-1} solids) ± SD**	***E. coli* K99 strain (CFU Log_{10} g^{-1} solids) ± SD**
80ºC Outlet air temperature		
Inoculated Plasma	7.31 ±0.39	7.66 ± 0.11
SDP*	<1	<1
70ºC Outlet air temperature		
Inoculated Plasma	6.93 ± 0.5	7.44 ± 0.42
SDP*	<1	<1

*SDP: Spray-dried plasma

4.4 Discussion

In this study, UV-C inactivation kinetics of two strains of *E. coli* from porcine (K88) and bovine (K99) origins were very similar, although such kinetics fit better to different models, as indicated by the

lower RMSE in each case. For both strains of *E. coli*, a rapid decrease in bacterial count was observed between 0 and 3000 J/L of UV-C, with the appearance of a residual population (Nres) afterwards. These results agree with other UV-C inactivation studies performed with *E. coli* (Hijnen, 2006). The reduction of the inactivation rate at high UV fluencies (tailing), could be caused by microorganism aggregation, appearance of a resistant subpopulation, hydraulic design (Hijnen, 2006), matrix effect or particle size effect (Winward, 2008). Porcine plasma is a dense, colored, liquid matrix with 8 to 10% solids, and contains a complex blend of different proteins with some of the proteins having binding properties (Burnouf, 2007). Therefore, the matrix and particle size effects of porcine plasma may have had a special impact on the tailing effects of UV-C treatment in the present study.

The residual population of *E. coli* after UV-C treatment should apparently be eliminated in the subsequent spray-drying process based on the total inactivation results by the spray-drying methods at the two outlet temperatures tested (Table 6). The outlet spray-drying temperature is 80ºC for commercial manufacturing of SDP (Pérez-Bosque *et al.*, 2016) and results of the present study suggest that both *E. coli* strains are very susceptible to spray-drying even at a lower outlet temperature (70°C). These results provide confidence that current commercial spray-drying conditions are highly effective for inactivation of *E. coli*.

Processing steps should be able to remove or inactivate a wide range of pathogens, according to the World Health Organization (WHO, 2004) guidelines on viral inactivation and removal procedures intended to assure the viral safety of human blood plasma products. These guidelines recommend that two or more robust, effective and reliable processes will be able to remove or inactivate 4 log_{10} or more of viruses.

Although the inactivation of viruses has to be considered separately, these guidelines used for virus safety in human plasma transfusion products can be applied to pathogens in general that affect animal blood then UV-C light treatment at 3000 J/L and spray-drying can be considered two robust safety procedures in the production of SDP since both methods individually inactivated at least 4 $\log_{10}$ *E. coli*.

In addition, the manufacturing process of SDP has other safety features, such as blood collection from healthy animals declared fit at slaughter for human consumption, pooling of inherent neutralizing antibodies (NA) against potential pathogens, and post-packaging storage in a dry environment at room temperature for at least 14 days (Pérez-Bosque *et al.*, 2016). Plasma pooling is also a recognized safety step in the production of certain human plasma products (Solheim *et al.,* 2000; 2006, 2008), since there is successful neutralization of antigens in the presence of NA. Some microorganisms in dehydrated form and stored under appropriate constant conditions can remain viable in a unique vitrified state for very long times, even years (Perdana *et al.*, 2013). Spray-dried plasma has a water activity of < 0.6 and is packaged and stored in mild temperatures. The storage conditions for SDP held at room temperature (~20ºC) for 14 days has been demonstrated effective to inactivate *Porcine epidemic diarrhea virus*, *Porcine reproductive and respiratory syndrome virus* and other coronaviruses when these viruses were experimentally inoculated on spray-dried plasma (Pujols and Segalés, 2014; Sampedro *et al.*, 2015). However, it is unknown if these storage conditions affect *E. coli* or other bacteria survival in spray-dried plasma. In the present study, the SDP storage temperature effect (20ºC for 14 days) on *E. coli* survival was not tested because both *E. coli* strains did not survive the spray-drying process. All the above-mentioned safety

features involved in the manufacturing process of SDP use different inactivation mechanisms, and collectively ensure the biosafety of SDP.

In conclusion, this study provides evidence that affordable levels of UV-C treatment (3000 J/L) of liquid porcine plasma can significantly decrease *E. coli* bacterial counts (4 log_{10}/mL at 3000 J/L). Furthermore, the study indicated that both UV-C treatment and spray-drying as independent safety procedures are very effective for inactivating *E. coli* K88 and K99. This novel UV-C technology can be adapted to further enhance the robustness of the manufacturing process for assuring the bio-safety of spray-dried plasma.

Chapter 5

Evaluation of the Effectiveness of the SurePure Turbulator^{TM} Ultraviolet-C Irradiation Equipment on Inactivation of Different Enveloped and Non-Enveloped Viruses Inoculated in Commercially Collected Liquid Animal Plasma

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5.1 Introduction

The objective of this study was to assess inactivation efficiency of the SurePure Turbulator™ UV-C irradiation system with selected swine enveloped viruses *Pseudorabies virus* (PRV), *Porcine reproductive and respiratory syndrome virus* (PRRSV), *Porcine epidemic diarrhea virus* (PEDV), *Bovine viral diarrhea virus* (BVDV), *Swine influenza A virus* (SIV) and *Classical swine fever virus* (CSFV) and non-enveloped viruses *Porcine parvovirus* (PPV), *Swine vesicular disease virus* (SVDV), *Porcine circovirus 2* (PCV-2) and *Senecavirus A* (SVA) inoculated in liquid bovine or porcine plasma. These viruses were selected because of their economic impact in livestock production and as models of viruses from different families with various genome type and size.

5.2 Material and methods

5.2.1 Viral strains and culture conditions

5.2.1.1 Pseudorabies virus

Pseudorabies virus strain NIA3 (McFerran *et al.*, 1979), kindly supplied by Joan Plana (Fort Dodge Veterinaria, Vall de Bianya, Spain), was propagated in the PK-15 cell line (provided by the Institute of Virology (UE and OIE Reference Laboratory for CSFV, Hannover), using a standard growth media (SGM) containing minimum essential medium eagle (MEM-E; ThermoFisher, Waltham, MA, USA) supplemented with 1% penicillin 10,000 U/mL and streptomycin 10 mg/mL (ThermoFisher), 0.5% Nystatin 10,000 IU/mL (Sigma-Aldrich), 1% L-glutamine 200 mM (ThermoFisher) and 5% of heat inactivated

fetal bovine serum tested free for virus and antibodies against pestiviruses (FBS; BioWest, Miami, FL, USA). PK-15 cells were grown in 175-cm^2 flasks (Corning, Corning, NY, USA), and when cells were confluent, the medium was discarded and adsorption was performed at a MOI 0.01. Virus stock was produced in the same cells to obtain 84 mL with a viral titer of $10^{8.95}$ $TCID_{50}$/mL that was used to inoculate 24 L of bovine plasma, achieving a final viral titer of $10^{6.5}$ $TCID_{50}$/mL.

5.2.1.2 Porcine reproductive and respiratory syndrome virus

Porcine reproductive and respiratory syndrome virus VP21 strain (Diaz *et al.*, 2005) was propagated in the Marc-145 cell line (ATCC CLR12231) grown in SGM with 5% FBS. Cells were cultured in 75-cm^2 flasks. When cells were confluent, the media was discarded and the adsorption was done using the virus at a MOI 0.01. After 1.5 hours at 37ºC, inoculum was removed and 30 mL of medium were added. This procedure was repeated until achieving 1,800 mL of viral suspension with a titer of $10^{5.57}$ $TCID_{50}$/mL that was used to inoculate 24 L of bovine plasma achieving a final viral titer of $10^{4.42}$ $TCID_{50}$/mL.

5.2.1.3 Porcine epidemic diarrhea virus

Porcine epidemic diarrhea virus CV777 strain (Debouck and Pensaert, 1980), kindly provided by Dr. Hans Nauwynck (University of Ghent, Belgium), was propagated in VERO cells (ATCC CCL-81) grown in SGM with 10% FBS. Cells were cultured in 175-cm^2 flasks and when they were confluent, the media was removed and cells were rinsed twice with PBS. Finally, inoculum was added at MOI 0.001 and adsorption was done for 1 hour at 37ºC. Subsequently, the inoculum was

discarded, flasks were rinsed twice with PBS and MEM-E was supplemented with 1% penicillin 10,000 U/mL and streptomycin 10 mg/ml (ThermoFisher), 0.5% Nystatin (10,000 U/mL), 1% L-glutamine (200 mM), 0.05% trypsin and 0.3% tryptose. The viral stock was produced in the same cells to obtain 2,110 mL of suspension with a viral titer of $10^{5.42}$ $TCID_{50}$ /mL that was used to inoculate 24 L of bovine plasma achieving a final viral titer of $10^{4.7}$ $TCID_{50}$ /mL.

5.2.1.4 Bovine viral diarrhea virus

Bovine viral diarrhea virus NADL was provided by the Institute of Virology (UE and OIE Reference Laboratory for CSFV, Hannover) and was propagated in MDBK cells (provided by the Institute of Virology (UE and OIE Reference Laboratory for CSFV, Hannover), grown in SGM with 5% FBS tested free of pestivirus antibodies. Cells were cultured in 175-cm^2 flasks. Media was discarded and the adsorption was done on confluent cells using BVDV strain at a MOI 0.01. After 1.5 hours at 37ºC, inoculum was removed and 50 mL of medium were added. This procedure was repeated until getting 255.3 mL of a suspension with a titer of $10^{7.87}$ $TCID_{50}$/mL that was used to inoculate 24 L of porcine plasma achieving a final viral titer of $10^{4.5}$ $TCID_{50}$/mL.

5.2.1.5 Classical swine fever virus

Classical swine fever virus strain Alfort 187, provided by the Institute of Virology (UE and OIE Reference Laboratory for CSFV, Hannover), was propagated in the PK-15 cell line (provided by the same Institute of Virology, Hannover), grown in SGM supplemented with 5%

of FBS free from pestivirus antibodies. A total of 160 mL of virus stock solution was produced in the same cells at a MOI 2, for a virus titer of $10^{7.36}$ $TCID_{50}$ /mL that was used to inoculate 24 L of bovine plasma achieving a final viral titer of $10^{5.19}$ $TCID_{50}$ /mL.

5.2.1.6 Swine influenza virus

Swine influenza virus strain H1N1 A/Swine/Spain/SF11131/ 2017 (Baratelli *et al.*, 2014) was propagated in MDCK cell line (ATCC CCL-34) grown in DMEM (ThermoFisher, Waltham, MA, USA) supplemented with 1% penicillin (10,000 U/mL), 1% streptomycin (10mg/mL; ThermoFisher), 0.5% Nystatin (10,000 U/mL; Sigma-Aldrich), 1% L-glutamine 200mM; ThermoFisher) and 5% FBS. Cells were cultured in 175-cm^2 flasks. When cells were confluent, the media was discarded and the adsorption was done at MOI 0.1. After 1 hour at 37ºC, inoculum was removed and 30 mL of medium were added. With this procedure, 300 mL of viral suspension with a final virus titer of $10^{7.2}$ $TCID_{50}$ /mL that was used to inoculate 24 L of bovine plasma achieving a final viral titer of $10^{5.3}$ $TCID_{50}$/mL.

5.2.1.7 Senecavirus A

Senecavirus A isolate BRA/UEL-PR/15 was kindly provided by Dr. Amauri Alfieri (Universidade Estadual de Londrina, Londrina, Brazil) and was propagated in PK-15 cell line (provided by the Institute of Virology (UE and OIE Reference Laboratory for CSFV, Hannover), grown in SGM with 10% FBS. Viral infection was done in confluent 175 cm^2 flasks at a MOI 0.001. After 1 hour of absorption, 50 mL of SGM

were added. CPE was visible at 72 h and the flasks were frozen. Following this procedure 1,100 mL of SVA with a titer of $10^{6.76}$ $TCID_{50}$/mL was produced, that was used to inoculate 24 L of bovine plasma achieving a final viral titer of $10^{5.43}$ $TCID_{50}$/mL.

5.2.1.8 Swine vesicular disease virus

Swine vesicular disease virus, strain UK-72, was provided by David K.J. Mackay (European Community Reference Laboratory for Foot and Mouth Disease, Institute for Animal Health. Pirbright, UK) to *Laboratorio de Sanidad Animal* (Barcelona). SVDV was propagated in SK-RST cell line (ATCC CRL-2842), grown in SGM supplemented with 5% FBS. A virus stock was produced using the same cell line at MOI 0.001 to obtain 24 mL of a virus stock solution with a titer of $10^{7.38}$ $TCID_{50}$ /mL that was used to inoculate 24 L of bovine plasma achieving a final viral titer of $10^{6.00}$ $TCID_{50}$ /mL.

5.2.1.9 Porcine circovirus 2

Porcine circovirus 2 genotype b isolate Sp-10-7-54-13 (Fort *et al.*, 2010) was cultured in the PK-15 cell line (provided by the Institute of Virology (UE and OIE Reference Laboratory for CSFV, Hannover), grown in SGM with 10% FBS. A mix of 6 mL of virus stock and 7 x 10^6 PK-15 cells resuspended in 50 mL of MEM-E (MOI 0.1) were added in 175 and 25 cm^2 flasks. At 24 hours cells were treated with glucosamine to facilitate the virus infection. Forty-eight hours later, viral infection was checked by IPMA (Rodríguez-Arrioja *et al.*, 2002) in the 25 cm^2 flask. If more than 25 positive cells were counted in a microscope field,

the 175 cm^2 flask was trypsinized and the cells were transferred to 3 new 175 cm^2 flasks. The process was repeated until 2,316 mL of virus stock with a titer of $10^{4.79}$ $TCID_{50}$ /mL that was used to inoculate 24 L of bovine plasma achieving a final viral titer of $10^{3.76}$ $TCID_{50\%}$ /mL.

5.2.1.10 Porcine parvovirus

Porcine parvovirus strain NADL-2 was kindly provided by Dr Albert Bosch (Department of Genetics, Microbiology and Statistics School of Biology, University of Barcelona, Spain). It was propagated in SK-RST cells (ATCC CRL-2842), grown in SGM supplemented with 5% FBS. One mL of virus stock and 9 mL of MEM-E supplemented with 1% pyruvate (Merck KGaA, Darmstadt, Germany) were added to a conical tube with 16 x 10^6 SK-6 cells and shaken for 30 minutes at 104 rpm and 37ºC. After that time, the contents of the tube were transferred to a 175 cm^2 flask, in which 40 mL of MEM-E supplemented with 1% pyruvate were added. This procedure was repeated until obtaining 811 mL of viral suspension with a titer of $10^{6.91}$ $TCID_{50\%}$ /mL that was used to inoculate 24 L of bovine plasma achieving a final viral titer of $10^{5.44}$ $TCID_{50}$ /mL.

Genomic data and virion size of each virus used in this study are displayed in Table 7.

Table 7: Virion size and genome characteristics of the viruses used in the study.

VIRUS	FAMILY	ENVELOPE	GENOME	SIZE (nm)	GENOME SIZE (Kb)
PRV	*Herpesviridae*	Yes	dsDNA	150-180	143.46
PRRSV	*Arteriviridae*	Yes	(+)ssRNA	50-65	15.43
PEDV	*Coronaviridae*	Yes	(+)ssRNA	95-190	28.03
BVDV	*Flaviviridae*	Yes	(+)ssRNA	25-120	12.57
CSFV	*Flaviviridae*	Yes	(+)ssRNA	25-120	12.3
SIV	*Orthomyxoviridae*	Yes	(-)ssRNA	80-120	13.15
SVA	*Picornaviridae*	No	(+)ssRNA	30	7.31
PPV	*Parvoviridae*	No	ssDNA	18-26	5.07
PCV2	*Circoviridae*	No	ssDNA	17	1.77
SVDV	*Picornaviridae*	No	(+)ssRNA	22-30	7.39

5.2.2 Plasma

Bovine and porcine blood was obtained from EU inspected slaughter facilities from animals inspected and approved for slaughter for human consumption. Blood was collected in stainless steel containers with sodium phosphate as anticoagulant. Blood was refrigerated and transported to the APC Europe laboratory (APC-Europe S.L.U., Granollers, Spain) and plasma was separated by centrifugation. Plasma was frozen at -20ºC.

Prior to virus inoculation and UV-C irradiation, plasma was thawed and filtered to eliminate potential cryoprecipitate. Before virus inoculation, a 100-mL sample of plasma was stored at –80ºC to determine absence of the test virus and absence of neutralizing antibodies to the test virus.

For all viruses tested, after virus inoculation to plasma, the 24 L mixture was divided into three equal 8 L aliquots. Each aliquot was subjected to UV-C irradiation to provide triplicate analytical results.

5.2.3 Virus inoculation in plasma procedure

Because antibodies against porcine viruses are not expected to be found in bovine blood, bovine plasma was inoculated with PRV, PRRSV, PEDV, CSFV, SIV, SVA, SVDV, PPV and PCV-2. Subsequent test confirmed the bovine plasma was negative for specific antibodies against the test virus. Similarly, porcine plasma was tested for BVDV antibodies by neutralizing peroxidase monolayer assay.

5.2.4 Settings of UV-C system

The setting of pilot scale UV-C used is described in Annex I. The UV-C dosage is expressed as J/L. The operation time of the UV-C treatment is based on the quantity of product to be treated and the flow rate of the product feed. At a flow rate of 4000 L/h, 9 s are required for 10 L of product to pass through the reactor once; thus, one turn of the product through the system is equivalent to a UV-C dose of 22.95 J/L. The UV dosage per L of liquid treated for one reactor with continuous flow was calculated as follows: Dosage = Total UV-C output per unit (W) / Flow rate (L/s) = 25.50 W / 1.11 L/s = (25.50 J/s)/ (1.11 L /s) = 22.95 J/L. Using an ammeter, the input current to the machine and its consumption were checked and it was established that they were appropriate according to the technical specifications and the data sheet of the UV-C lamp provided by SurePure.

A standard CIP process as described by Keyser et al. (2008) based on a treatment with NaOH 5%, was implemented prior to and following each UV-C treatment.

5.2.5 Sampling

Plasma flow was stabilized at 4000 L/h with the UV lamp switched off. After 5 minutes of stable flow, a positive control (0 J/L) sample was collected into sterile container. Then, the UV-C lamp was switched on and irradiation started. 175 mL of treated plasma were collected into sterile containers at different UV-C doses (750, 1500, 3000, 6000, and 9000 J/L).

5.2.6 Analysis of viral titration

Infectivity of samples was determined in target cell cultures using the microtiter assay procedure (Kasza *et al.*, 1972). The microtiter assay was done in a 96-well plate for all tested viruses, except for PEDV. Titration of virus was done using the whole plate for every dilution, from -1 to -5 dilutions, to amplify the detection capability of the test. Final titer was expressed as log $TCID_{50}$/mL. Once all samples were titrated and the first negative dose was found, further steps were developed to ensure full inactivation of the samples. First, 50 mL of this negative dose sample were inoculated into 10 175-cm^2 culture flasks and incubated at 37ºC for 5 days. The flasks were frozen and thawed three times. The liquid was then centrifuged at 3000 rpm and 25 mL were inoculated into 5 175-cm^2 flasks. The flasks were incubated 5 days at 37ºC. The procedure was repeated again and, finally, only one flask was inoculated with 5 mL and incubated at 37ºC for 5 days. If the final flask was negative, it was considered that no viral particles were present in the initial sample; if the final flask was found positive, a titration of the plasma was repeated again on ten 96-well plates to analyze a total volume of 50 mL.

Due to the cytotoxic effect of the plasma, strong washes to eliminate serum used to propagate the cell culture and trypsin addition, PEDV was titrated by means of PFU/mL. A 12-well culture plate with 100% confluency was inoculated with diluted plasma in MEM supplemented with 0.05% trypsin and 0.3% tryptose, but without FBS. Whole plates were used for each dilution, from -1 to -5, to amplify the detection capability of the test. Conversion to $TCID_{50}$/mL was done multiplying PFU x 0.7

(https://www.lgcstandardsatcc.org/support/faqs/48802/Converting+TCID50+to+plaque+forming+units+PFU-124.aspx?geo_country=es). Negative samples were inoculated to 175-cm^2 culture bottles to analyze a total volume of 50 mL (10 mL by bottle), and passaged three times before being discarded as negative. If a sample was found positive, a titration of pure plasma was repeated on 10 12-well plates to analyze the same total volume of 50 mL. This procedure was intended to increase 10 times the initial analyzed volume.

5.2.7 Modeling of inactivation

Microbial inactivation due to thermal and non-thermal processes can be represented by eight possible curves (Geeraerd *et al.*, 2005). The GInaFiT software was used to test linear and non-linear survival curves (Geeraerd *et al.*, 2005), using the biphasic (Cerf, 1977),Weibull (Mafart *et al.*, 2002), Weibull plus tail (Albert and Mafart, 2005) and biphasic plus shoulder (Geeraerd *et al.*, 2005) models. The GInaFiT software has been used to test survival kinetics of different bacteria and viruses when submitted to heat treatment or UV-C irradiation (Orlowska *et al.*, 2015).

These equations are described in Chapter 1: Introduction, section 1.11.4 Inactivation kinetics.

Using these equations, the software automatically calculates the 4D value (the dose needed to inactivate 4 log_{10} of viral load). In the case of BVDV, since the titer expressed as Log_{10} $TCID_{50}$/mL did not achieve 4 log_{10}, the 4D value was calculated for Log_{10} $TCID_{50}$ in 10 mL of tested sample. In the case of PCV2, the 4D value could not be calculated due to the process did not achieve 4 log_{10} inactivation.

5.2.8 Statistical analyses

Data were expressed as the mean as Log_{10} $TCID_{50}$ with standard deviations of three independent replicates. Mean, standard deviations, ANOVA and F-Test for comparisons were calculated with Excel 2007 (Microsoft Office). The Tukey test was done to determine significant differences between UV-C radiation doses. Mean square error (MSE), goodness of fit in terms of root mean square error (RMSE), correlation coefficient (R^2) and adjusted correlation coefficient (adj-R^2) values were calculated with the GInaFiT software (Geeraerd *et al.*, 2005). The inactivation model with the best fit corresponded to the model with the smallest RMSE (Geeraerd *et al.*, 2005).

5.3 Results

Bovine plasma used in the studies was negative for viral contamination or neutralizing antibodies against tested viruses (PRV, PRRSV, PEDV, CSFV, SIV, SVA, SVDV, PPV and PCV-2). In addition, porcine plasma was free from BVDV and BVDV antibodies.

The results of UV-C viral inactivation are summarized in Table 8 and in Figures 9, 10 and 11.

In general, all enveloped viruses tested were inactivated at <3000 J/L and showed non-linear inactivation kinetics, biphasic or Weibull distributions, with low RMSE values resulting in 4D values under 3000 J/L (Fig. 9, Fig.10 and Table 8). The two best non-linear inactivation kinetics with the smallest RMSE are included in Table 8.

Greater ranges of stability were found for non-enveloped viruses. PPV was inactivated at <3000 J/L, but other non-enveloped viruses such as SVA, SVDV and PCV-2 required a greater UV-C dose to be inactivated (Table 8; Fig. 11). For example, all three samples of SVDV at 6000 J/L were negative by the microtiter assay. However, the subsequent 3 blind passages performed for each 6000 J/L sample were positive. Afterwards, a titration of 25 mL was done by duplicate, obtaining one positive out of 960 wells, so, it was concluded that there were 0.02 particles/mL (0.014 TCID50%) in the 6000 J/L samples (1 particle/50 mL assayed volume). To further confirm the dose required to inactivate SVDV, three additional blind passages were performed for the three samples irradiated at 9000 J/L, and one was found positive. Titration of the original sample of 9000 J/L (25 mL) was done; one out of 480 inoculated wells was positive. It was calculated that 0.04 particles per milliliter (0.028 TCID50%) were still present in the only positive sample of 9000 J/L (1 particle/25 mL assayed volume). The most resistant virus was PCV-2, since the total log10 reduction achieved was 2.71 $\log_{10}$ at 9000 J/L.

Table 8: Log reduction of viral titers expressed as Log_{10} $TCID_{50}$ at different UV-C doses and statistical parameters of models for inactivation of enveloped or non-enveloped viruses.

Parameter	PRV	PRRSV	PEDV	BVDV†	SIV	CSFV	SVDV	PCV-2	PPV	SVA
Theoretical viral titer in 24 L	6.5	4.42	4.7	4.5	5.3	5.19	6	3.26	5.44	5.43
Viral titer (VT) before UV-C irradiation	4.53±0	4±0.1	4.07±0.11	4.16±0.24	5.09±0.05	4.09±0.02	4.08±0	3.26±0.12	4±0.06	4.68±0.18
VT at 750 J/L	3.78±0.49	1.33±0.08	2.02±0.42	3.36±0.13	4.06±0.05	3.23±0.13	4.08±0.04	1.68±0.04	3.23±0.12	4.12±0.06
VT at 1500 J/L	1.36±0.07	BDL	1.19±0.35	1.63±0.08	1.65±0.06	0.64±0.03	1.69±0.04	1.54±0.05	1.69±0.04	2.99±0.06
VT at 3000 J/L	0.02±0.01	BDL	BDL	BDL	BDL	BDL	1.46±0.01	1.48±0.01	BDL	0.94±0.1
VT at 6000 J/L	BDL	BDL	BDL	BDL	BDL	BDL	0.02±0.01	0.73±0.04	BDL	BDL
VT at 9000 J/L	BDL	BDL	BDL	BDL	BDL	BDL	0.04±0.02	0.55±0.01	BDL	BDL

[1] BDL: Below Detection Limit, being the limit of detection for each batch 1/50 mL = 0.02 viral particles/mL [2]MSE: Mean Sum of Squared Error. [3] RMSE: Root Mean Sum of Squared Error. This parameter determines the model that best fits the data. NC: Not able to calculate due to the initial titer lower than 10^4 $TCID_{50}$/mL. †Viral titer calculated for 10 mL of analysed sample.

Table 8 (continuation): Log reduction of viral titers expressed as Log 10 $TCID_{50}$ at different UV-C doses and statistical parameters of models for inactivation.

Parameter	*PRV*	*PRRSV*	*PEDV*	*BVDV†*	*SIV*	*CSFV*	*SVDV*	*PCV-2*	*PPV*	*SVA*
Best Fit model	Weibull + tail	Log linear	Biphasic	Weibull	Biphasic + shoulder	Biphasic + shoulder	Log linear + tail	Biphasic	Log linear + shoulder	Biphasic + shoulder
MSE[1]	0.0195	0.0238	0.0662	0.0193	0.0023	0.0049	0.3195	0.0144	0.0054	0.0099
RMSE[2]	**0.1398**	**0.1543**	**0.2574**	**0.1389**	**0.0478**	**0.0703**	**0.5653**	**0.1199**	**0.0733**	**0.0993**
R-Square	0.9978	0.9966	0.9898	0.9979	0.9998	0.9994	0.9499	0.9855	0.9992	0.9988
R-Square adjusted	0.997	0.9961	0.9859	0.9975	0.9997	0.9991	0.9432	0.9824	0.999	0.9983
4D reduction (J/L)	1612	1004	1953	1943	1639	1641	3708	NC	2161	3223
Second Best Fit model	Biphasic + shoulder	Weibull	Weibull	Log linear + shoulder	Log linear + shoulder	Weibull	Weibull + tail	Weibull	Weibull	Biphasic
MSE[1]	0.0223	0.0272	0.0834	0.0389	0.038	0.1955	0.3423	0.0233	0.0076	0.0395
RMSE[2]	**0.1494**	**0.165**	**0.2888**	**0.1973**	**0.1948**	**0.4421**	**0.585**	**0.1528**	**0.0873**	**0.1988**
R-Square	0.9978	0.9967	0.9855	0.9959	0.9958	0.9717	0.9499	0.9748	0.9988	0.9947
R-Square adjusted	0.9965	0.9955	0.9823	0.9949	0.9949	0.9654	0.9392	0.9714	0.9985	0.9932
4D reduction (J/L)	1786	1004	1953	1861	1723	1872	3708	NC	2190	3050

[1] BDL: Below Detection Limit, being the limit of detection for each batch 1/50 mL = 0.02 viral particles/mL [2]MSE: Mean Sum of Squared Error. [3] RMSE: Root Mean Sum of Squared Error. This parameter determines the model that best fits the data. NC: Not able to calculate due to the initial titer lower than 10^4 $TCID_{50}$/mL. †Viral titer calculated for 10 mL of analysed sample.

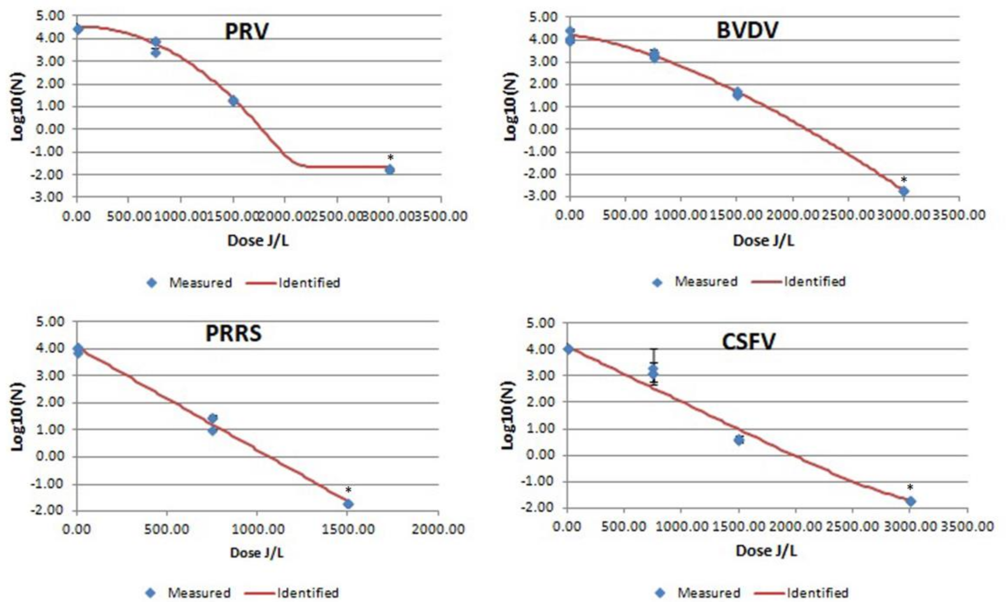


Fig. 9. Mean PRV, PRSSV, CSFV and BVDV log_{10}/mL values after UV-C irradiation of bovine plasma at different UV irradiation doses. Blue diamonds indicated measured results of the viral titer at different UV-C irradiation doses expressed as mean log_{10}/mL (n=3 replicates). Red line is the identified inactivation curve model. *: indicates a value below the detection limit.

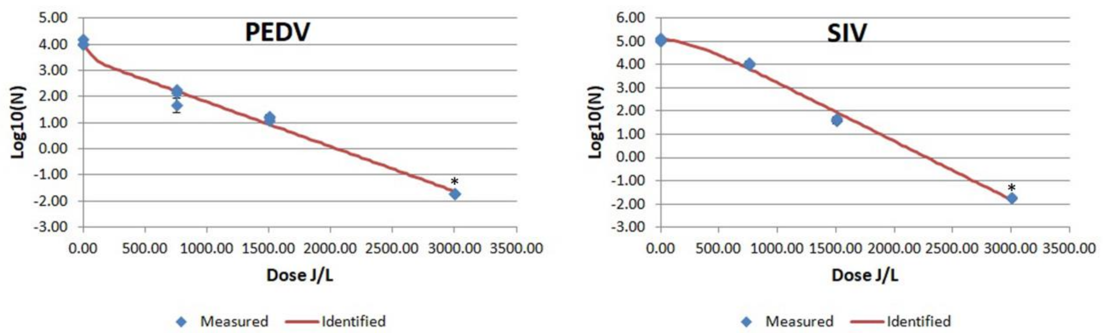


Fig. 10. Mean PEDV and SIV $\log_{10}$/mL values after UV-C irradiation of bovine plasma at different UV irradiation doses. Blue diamonds indicated measured results of the viral titer at different UV-C irradiation doses expressed as mean $\log_{10}$/mL (n=3 replicates). Red line is the identified inactivation curve model. *: indicates a value below the detection limit.

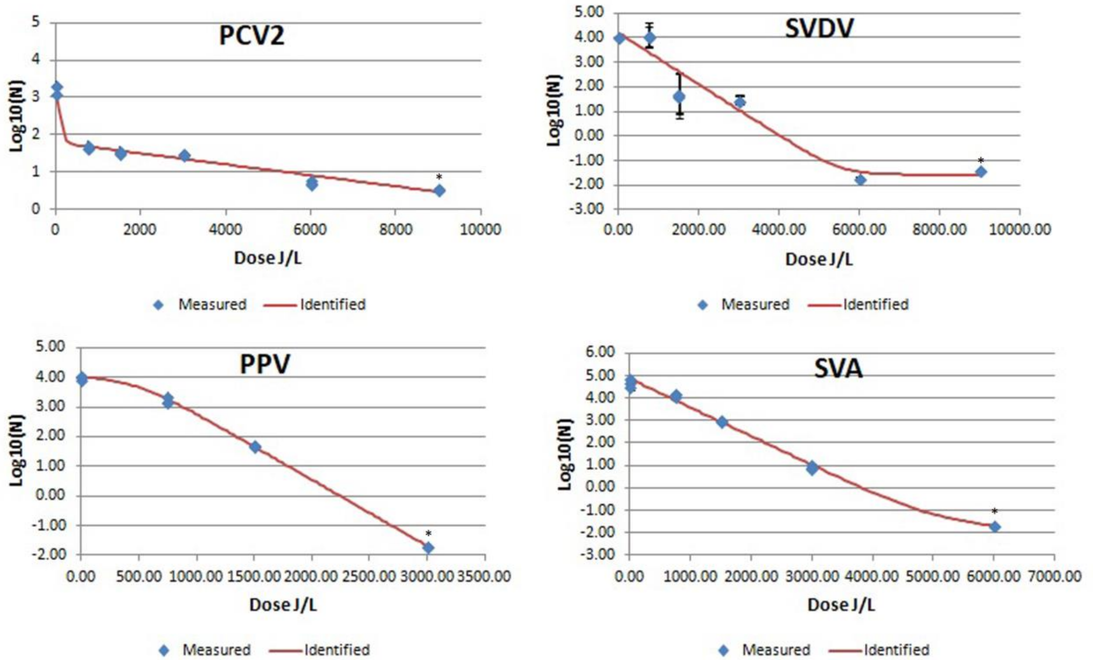


Fig. 11. Mean PCV-2, SVDV, PPV, and SVA log_{10}/mL values after UV-C irradiation of bovine plasma at different UV irradiation doses. Blue diamonds indicated measured results of the viral titer at different UV-C irradiation doses expressed as mean log_{10}/mL (n=3 replicates). Red line is the identified inactivation curve model. *: indicates a value below the detection limit.

5.4 Discussion

The efficacy of microbial reduction by UV-C treatment of liquids depends on different factors, including the microorganism used, the opaqueness of the liquid, the presence of suspended particles, and the microbial contamination level at the starting point (Groenewald *et al.*, 2013). Animal plasma from slaughterhouse is an opaque liquid; therefore, UV-C radiation does not penetrate this liquid. This problem

has been overcome by introducing turbulent flow achieving that the whole volume of liquid passes close to the UV-C radiation source (Wang *et al.*, 2004; Fredericks *et al.*, 2011; Seltsam & Müller, 2011; Simmons *et al.*, 2012; Alberini *et al.*, 2015; Chapter 3). The UV-C system used in this work can be expanded to efficiently process large volumes of liquid compatible with commercial production of fruit juices, wine (Keyser *et al.*, 2008; Fredericks *et al.*, 2011) and milk (Donaghy *et al.*, 2009). It is important to differentiate this unique design of the SurePure Turbulator system for treatment of high volume of opaque liquids from other UV systems developed for treatment of human plasma fractions (Wang *et al.*, 2004; Seltsam and Müller, 2011) that are designed for treatment of small plasma fraction bags under gentle agitation but cannot be scalable to the volumes used by the commercial SDAP industry.

A preliminary step to implement industrial plasma irradiation with UV-C consisted of demonstrating such treatment does not affect the functionality of proteins present in SDAP. Similar growth and health performance of pigs fed diets with UV-SDAP versus non-irradiated SDAP has been reported (Polo *et al.*, 2015; Cottingim *et al.*, 2017).

The next step was to investigate the inactivation capacity of the system on possible contaminating viruses in the plasma. For this purpose, selected viruses representing enveloped and non-enveloped, DNA and RNA, single-stranded and double-stranded viruses of importance for porcine industry were spiked into SDAP and irradiated. Furthermore, the different viruses tested may be considered surrogates for other untested viruses belonging to the same family or genus, although it is always recommended to test specifically each particular virus (Keil *et al.*, 2015).

In the present study, UV-C radiation was more effective inactivating tested enveloped viruses compared to the non-enveloped

viruses. Enveloped viruses could not be detected when the UV-C dose was >3000 J/L. The best fit lines describing inactivation kinetics of the enveloped viruses were curvilinear biphasic equations. This could be due to lack of more points of collection; in many cases the last data point(s) appeared to be beyond the minimum UV-C radiation required to inactivate the entire virus in the sample, creating an apparent curvilinear shape. Cutler et al. (2011) reported biphasic inactivation kinetics of PRRSV, BVDV and SIV using a different UV-C delivery system and with the virus suspended in culture media rather than blood plasma. Although almost a lineal response was observed in the present study, the best model for BVDV was Weibull, and biphasic plus shoulder for SIV. Nevertheless, in both studies, despite using two different irradiation equipment (here versus the used by Cutler et al. (2011)), the relative sensitivity of the virus to UV-C inactivation was similar with PRRSV being more sensitive than BVDV or SIV.

A total inactivation (3.16 log) of BVDV was achieved at 3000 J/L. The inactivation kinetics obtained with BVDV is very similar to the one observed in the other tested pestivirus, CSFV, which could indicate that members of the same genus could respond similarly to UV-C treatment.

PRV titer decreased more than 4 log at UV-C irradiation treatment < 3000 J/L, which represented a better inactivation ratio than that reported by another experiment using Riboflavin-UV photochemical based technology (Keil *et al.*, 2015). The degree of inactivation by UV-C irradiation may depend on the technology used (Hijnen, 2006).

To the authors' knowledge, this is the first study showing effective results in the inactivation of PEDV by means of UV-C.

The non-enveloped viruses that were tested appeared to be more resistant to inactivation by UV-C radiation. The inactivation curve for PPV was consistent with previously published data (Polo *et al.*, 2015), with apparent linear inactivation in response to increasing UV-C dose. Likewise, the inactivation curve for SVA appeared to be linear except for the highest UV-C dose. The inactivation curve for SVDV showed minimal inactivation at low UV-C dose, suggesting that a minimal UV-C dose was necessary before virus was inactivated. This has been reported by others (Geeraerd *et al.*, 2005) who suggested that for some virus inactivation does not occur until a minimum UV-C dose is achieved. However, in this experiment appears to be another shoulder in the inactivation curve at intermediate UV-C dosage (1500 and 3000 J/L). Furthermore, while the highest doses appeared to inactivate all the virus, subsequent passages detected very low residual viral infectivity. The inactivation curve for PCV-2 demonstrated that UV-C radiation inactivated this virus; however, even at the highest dose (9000 J/L), residual levels of PCV-2 were recovered. The presence of a resistant viral subpopulation has been attributed to the ability of some viruses to take advantage of host cell repair mechanisms (Freeman *et al.*, 1987), or in some cases they can code for their own repair machinery (Srinivasan *et al.*, 2001). It has also been argued that viral particle clumping, particularly with other cells or debris, may shield some virus from UV-C radiation (Cutler *et al.*, 2011).

In the present study, a difference between the theoretical titer in the spiked plasma and the measured titer at time zero was observed. The difference was greater with enveloped viruses than with non-enveloped viruses. Generally, enveloped viruses are more sensitive to environmental conditions such as salts, temperature changes, freezing

and thawing or presence of anticoagulants (WHO, 2004) and could explain the reduction in titer before irradiation compared to the theoretical titer (Polo *et al.*, 2005).

There was not a clear relationship between rate of inactivation and genome type or size. Theoretically, DNA is more sensitive to UV-C due to the presence of thymine (Rauth, 1965). In contrast, DNA repair can reduce the UV-C effect, especially for dsDNA viruses (Lytle *et al.*, 1972). In the present study, PRV (a dsDNA virus) had 4D reduction estimated at 1612 J/L, which was not apparently different from the 4D reduction values obtained for other tested RNA viruses (PEDV and SIV). Obtained results with viruses evaluated in this study suggest that UV-C inactivation susceptibility of both types of viral genome was similar.

It has been suggested that the longer the genome, the higher the susceptibility to UV-C damage (Lytle and Sagripanti, 2005). However, based on the present results, increased genome size did not appear to increase virus susceptibility to UV-C radiation when spiked into commercially collected animal plasma and exposed to UV-C. Only a weak linear trend (r^2=0.43) was found in the current data when comparing genome size to the 4D of viruses (without outlier's results PEDV and PRV). Curiously, Wang et al. (Wang *et al.*, 2004) found that viruses with large genomes (*Adenovirus* and *Reovirus*) were inactivated with higher UV-C doses than viruses with shorter genomes. These authors indicated that their unexpected survival curves compared with other UV-C irradiation studies was difficult to explain, and suggested differences in the shape, size and lamp geometry of UV-irradiation systems as well as in protein concentrations and composition of process streams to explain their results. Also, genome configuration may affect the virus sensitivity to UV-C radiation. For example, both PPV and PCV-

2 have a relatively small genome size (5.07 and 1.77 Kb, respectively). However, estimated 4D for PPV was 2161 J/L while that for PCV-2 was >9000 J/L. It is possible that the linear configuration of PPV genome could be more easily damaged, whereas the circular genome of PCV-2 may provide resistance to the formation of thymidine dimers by UV-C radiation. In any case, the present study should be considered exploratory in nature regarding the relationship between size and type of the genome and susceptibility to inactivation by UV-C irradiation.

In addition to the photochemical damage of UV-C irradiation on nucleic acids, UV-C also induces reactive oxygen species that may interact with the external lipid bilayer membrane of enveloped viruses (Chan *et al.*, 2006), causing lipid peroxidation (Phukan *et al.*, 2018). This effect may further explain why enveloped viruses were very susceptible to UV-C irradiation. In fact, in the present study, irradiation doses used were between 30-375 times higher than those described in the literature (Meunier *et al.*, 2017). These differences could be due to the presence of proteins and other molecules in solution, to the possibility of virus clumping with these molecules, to the greater opacity of the liquid against UV, to the higher basal absorbance and/or to the darker color of the liquid plasma solution compared to other more transparent solution like water or other buffers like PBS. Nevertheless, our results are closer to irradiation values found in other opaque raw materials like milk or juices (Fredericks *et al.*, 2011; Crook *et al.*, 2015).

Overall, the SurePure UV-C Turbulator design was effective in inactivating a wide variety of virus spiked into commercially collected liquid animal plasma. Data from the present study indicated that the UV-C irradiation of liquid plasma is a technology that could provide an additional inactivation step to the industrial production process for

SDAP. Exposure to UV-C is extensively used for the disinfection of liquid media and surfaces due to its germicidal activity (Guerrero-Beltran and Barbosa-Cánovas, 2004; Lin *et al.*, 2012). Therefore, UV-C irradiation of liquid plasma during the manufacturing process for SDAP has a potential application for inactivation of microbial contaminants without causing negative effects on the nutritional or physical qualities of the treated material (Hijnen, 2006; Dee *et al.*, 2011). Furthermore, the UV-C mechanism of inactivation (DNA or RNA damage) is different than the thermal inactivation provided by the spray-drying step. In consequence, the UV-C step can be considered independent from the spray-drying process and may be a synergistic process as both procedures have different inactivation targets.

In conclusion, obtained results demonstrate UV-C as a suitable technology to be applied in the manufacturing process of SDAP as a redundant biosafety step for the inactivation of viruses of concern for the livestock industry.

Chapter 6

Use of a swine bioassay to evaluate the ability of UV-C irradiation to inactivate potential pathogens in commercially collected porcine plasma

Submitted to publication

6.1 Introduction

It is important to validate individual inactivation steps that are included in commercial production processes for SDPP (WHO, 2004). A swine bioassay including intraperitoneal injection of a sample into naïve pigs with a test material represents an extremely sensitive method to test infectivity (Christopher-Hennings, *et al.*, 2012). In a swine bioassay, it is important to monitor the pigs after injection with the test material for viremia and/or seroconversion, which may indicate the presence of infective virus in the test material. This procedure is considered a very sensitive method to test the safety of a specific product such as porcine plasma (Sampedro *et al.*, 2015) and can determine if genome detected by PCR analyses can cause infectivity.

In addition, commercial quantitative real time PCR (qRT-PCR) test kits are able to detect small amounts of pathogenic microorganisms in a sample. However, PCR methods are unable to differentiate between infective or non-infective presence of microorganisms. Although infectiousness cannot be assessed by means of these molecular methods, the larger the amplicon to be detected, the higher the likelihood of DNA/RNA integrity. Therefore, it would be of interest to explore the design of PCRs amplifying longer sequences (in opposition to PCR diagnostic kits with high sensitivity based on the detection of small amplicons). Thus, a potential better correlation between integrity of genome and infectivity may be established. Anyway, even a complete genome is not always indicative of infectivity because the pathogen can be damaged without affecting the genome.

The objective of the present study was to determine if UV-C irradiation of liquid commercially collected porcine plasma by a

specifically designed UV system developed for large volumes of opaque liquids (SurePure Turbulator) could inactivate infective virus as measured in a pig bioassay. The primary aim was to evaluate inactivation of *Porcine circovirus 2* (PCV-2) and *Porcine reproductive and respiratory syndrome virus* (PRRSV) because it is not uncommon for commercially collected plasma to be qRT-PCR positive for these economically important viruses of concern for the global swine industry. Furthermore, the presence and possible transmission of other potential contaminating agents of importance for the swine industry, such as *Transmissible gastroenteritis coronavirus* (TGEV), *Swine Influenza A virus* (SIV), *Porcine parvovirus* (PPV), *Porcine epidemic diarrhea virus* (PEDV), *Swine rotavirus A* (RVA), *Bovine viral diarrhea virus* (BVDV), *Border disease virus* (BDV), *Hepatitis E virus* (HEV) and *Salmonella enterica* were evaluated. Another objective was to develop PCR methods yielding longer sequence amplicons for PCV-2 and PEDV and test the virus genome disruption effect of different UV irradiation doses on these two pathogen signals, compared with standard commercial qRT-PCR kits that use shorter sequence amplicons.

6.2 Materials and methods

6.2.1 Plasma selection

Ten 10-L batches of liquid porcine plasma collected from a commercial abattoir (each batch of plasma was collected from a plasma pool from ~10,000 pigs) were frozen (-20ºC) prior to pre-screen testing for PCV-2 and PRRSV genome and antibodies. The test batch for use in the UV-C test was selected based on the highest number of PCV-2 DNA

copies measured by real-time quantitative PCR (qRT-PCR) using a test kit (LSI VetMAX^{TM} Porcine Circovirus Type 2 Quantification, Thermo Fisher Scientific, Massachusetts, USA) and the lowest level of PCV-2 antibodies analyzed by ELISA (Ingezim Circo IgG, 11.PCV.K.1/5 ELISA, INGENASA, Madrid, Spain) among the pre-screened liquid plasma batches. PRRSV RNA was also detected and quantified by a reverse transcriptase qPCR (qRT-PCR) method (LSI VetMAX^{TM} PRRSV EU/NA Real-Time PCR Kit. Thermo Fisher Scientific, Massachusetts, USA) and presence of PRRSV antibodies was analyzed with a commercial ELISA test (IDEXX PRRS X3 Ab Test ELISA, IDEXX, Hoofddorp, The Netherlands).

6.2.2 Plasma UV-C irradiation

Prior to UV-C irradiation, the selected plasma batch was thawed and filtered to eliminate potential cryoprecipitate.

The UV-C reactor SP1 produced by Sure Pure Operation AG (Zug, Switzerland) was used and its settings are described in Annex 1.

Samples were taken after exposure to 0 J/L (initial sample), 3000 J/L and 9000 J/L energy from the UV-C source, as previously described (Chapter 3, 4 and 5). One-liter samples of plasma were irradiated with 0 J/L, 3000 J/L and 9000 J/L UV-C and 10 mL were used for intraperitoneal injection in naïve pigs.

6.2.3 Animals and housing

At approximately 14 days of age, blood samples of selected pigs were collected at the farm of origin to verify a negative result for PRRSV antibodies and genome; in addition, these animals had low PCV-2 antibody ELISA S/P values (of maternal origin) and were negative for PCV-2 qRT-PCR. A total of 40 male piglets (25 ± 3 days of age; initial average body weight 5 ± 0.5 Kg) were transported and allocated at the *Institut de Recerca i Tecnologia Agroalimentàries* (IRTA) experimental farm in Alcarràs (Lleida, Spain), in individual rooms and separated from other animals for about three weeks before the start of the study. At the experimental farm, piglets were sampled at 35 and 45 days of age, and the experimental groups were established once piglets were proven seronegative by ELISA against PCV-2 and PRRSV at 50 days of age. Three pigs were unthrifty during this period and were excluded from the study; the remaining 37 pigs were weighed, ear-tagged and randomly distributed in five experimental groups of 6 to 8 pigs per group after matching weights between groups (7 pigs in negative control group, 8 pigs in each of the 3 treatment groups and 6 pigs in the positive control group). Each group of animals was allocated in separate boxes and also in different rooms, thus no air space was shared between groups. Each box had 7.5 m^2 of surface area for the pigs. Environmental conditions of rooms were maintained at 20-24ºC, and an area with a heat lamp source at 30-35ºC was included inside each box. Illumination consisted of natural light.

To ensure that no cross contamination between treatment groups or external contamination occurred, rooms were closed, air entry was regulated, and strict biosafety protocols for the caretakers were

implemented. Caregivers were trained to wear TYVEX overalls, overcoats, head coverings and gloves at the entrance of each room for daily animal care.

6.2.4 Experimental design and sampling

Group 1 (n=7) represented the negative control group and was injected with 10 mL of phosphate buffered saline (PBS) solution (Saline Solution Vitulia, ERN Laboratories, Barcelona, Spain). Group 2 (n=8) pigs were injected with 10 mL of native non-UV-C irradiated plasma (raw native liquid plasma). Group 3 (n=8) animals were injected with 10 mL of plasma UV-C irradiated at 3000 J/L. Group 4 (n=8) pigs were injected with 10 mL of plasma UV-C treated at 9000 J/L. Group 5 (n=6) was used as the positive control group and was injected with 10 mL of PCV-2 at a dose of 100 $TCID_{50}$/mL. All products/inoculum were administrated by intra-peritoneal route to all groups. Animal procedures were approved by the committee of ethics and welfare with the protocol approval number CEA-OH/9561/2.

Piglets were monitored daily for clinical signs of infection during a 50-day study period. Blood samples were collected on days 0, 15, 30 and 50 post-injection and tested for viremia and antibody seroconversion against PRRSV, PCV-2, TGEV, SIV, PPV, RVA, PEDV, BVDV, BDV, HEV and *S. enterica*.

At the end of the study, the animals were euthanized by an overdose of sodium pentothal (Euthasol 400®; Laboratorios ECUPHAR, Barcelona, Spain). All piglets were necropsied and samples of lung, tonsil, lymph node, bile and feces were collected for further analysis.

6.2.5 Porcine Circovirus 2 inoculum

Porcine Circovirus 2 genotype b isolate Sp-10-7-54-13 (Fort *et al.*, 2010) was titrated in PK-15 cell line. The PCV-2 viral suspension was adjusted to 100 $TCID_{50}$/mL with PBS solution to constitute the inoculum of the positive control group number 5.

6.2.6 Laboratory procedures

The selected plasma batch was tested for the presence of PCV-2 and PRRSV genome and antibodies, as indicated previously, and was also analyzed for the presence of other pathogens by commercial qPCR or qRT-PCR techniques, including TGEV (EXOone TGEV, EXOPOL, Zaragoza, Spain), SIV (EXOone Influenza A, EXOPOL, Zaragoza, Spain), PPV (EXOone Parvovirus, EXOPOL, Zaragoza, Spain), PEDV (EXOone PEDV, EXOPOL, Zaragoza, Spain), RVA (EXOone Rotavirus A, EXOPOL, Zaragoza, Spain), HEV (Path-HEV advance from Genesig-Primerdesign, Cambridge, UK), and *S. enterica* (EXOone Salmonella enterica, EXOPOL, Zaragoza, Spain). BVDV, and BDV were analyzed by previously published PCR techniques (Blázquez *et al.*, 2019).

Besides for PCV-2 and PRRSV, the presence of antibodies for the following agents were also evaluated in the plasma batch: TGEV (Ingezim TGEV, INGENASA, Madrid, Spain), SIV (CIVTEST SUIS INFLUENZA, Hipra, Amer, Spain), PPV (Ingezim PPV, INGENASA, Madrid,Spain), RVA (Ingezim Swine Rotavirus, INGENASA, Madrid, Spain), PEDV (Ingezim PEDV, INGENASA, Madrid, Spain), HEV (ID Screen Hepatitis E Indirect Multi-species, IDvet Genetics, Grabels,

France) and *S. enterica* (IDEXX Swine Salmonella Ab, IDEXX, Hoofddorp, The Netherlands). Antibodies against BVDV, BDV were evaluated by sero-neutralization tests, following the OIE protocols (www.oie.int).

After inoculation, animals were monitored for the development of viremia and antibodies to pathogens identified in the plasma inoculum (Table 10). Serum samples taken on 50 dpi were analyzed for the presence of antibodies against PCV-2, PRRSV, TGEV, SIV, PPV, RVA, PEDV, HEV and *S. enterica* with the same commercial kits mentioned above. If antibodies were found in the serum samples 50 dpi, the presence of virus was determined in the same serum samples (50 dpi) by qRT-PCR. Serum samples (50 dpi) were tested for PCV-2 and PRRSV by qRT-PCR.

Serum collected at 15, 30 and 50 dpi and feces (50 dpi) were tested for PPV and RVA. Bile (50 dpi) was tested for the presence of HEV RNA. SIV was analyzed (qRT-PCR) in serum and lung tissue (50 dpi) samples by qRT-PCR with the technique previously cited.

6.2.7 PCR analysis of genome integrity

In order to evaluate the effect of the UV irradiation on the viral genome integrity, PCV-2 and PEDV were chosen as a model. Specifically, a conventional PCR to amplify a large amplicon of PCV2 and PEDV genome was used in comparison with commercial qRT-PCR.

6.2.7.1 PCV-2

Eight liters of commercial liquid bovine plasma were inoculated with PCV-2 genotype b isolate Sp-10-7-54-13 (Fort *et al.*, 2010) and

irradiated at different irradiation doses 0, 750, 1500, 3000, 6000 and 9000 J/L following the procedure previously explained. The inoculum of PCV-2 genotype b isolate Sp-10-7-54-13 was obtained by culture in the PK-15 cell line provided by the Institute of Virology (UE and OIE Reference Laboratory for CSFV, Hannover), grown in Minimum Essential Medium Eagle (MEM-E; ThermoFisher, Waltham, MA, USA) supplemented with 1% penicillin 10,000 U/mL and streptomycin 10 mg/mL (ThermoFisher), 0.5% Nystatin 10,000 IU/mL (Sigma-Aldrich), 1% L-glutamine 200 mM (ThermoFisher) and 10% of heat inactivated fetal bovine serum tested free for virus and antibodies against pestiviruses (FBS; BioWest, Miami, FL, USA). A mix of 6 mL of virus stock and 7 x 10^6 PK-15 cells re-suspended in 50 mL of MEM-E (MOI 0.1) were added in 175 and 25 cm^2 flasks. At 24 hours, cells were treated with glucosamine to facilitate the virus infection. Forty-eight hours later, viral infection was checked by IPMA (Rodríguez-Arrioja *et al.*, 2002) in the 25 cm^2 flask. If more than 25 positive cells were counted in a microscope field, the 175 cm^2 flask was trypsinized and the cells were transferred to 3 new 175 cm^2 flasks. The process was repeated until 772 mL of virus stock with a titer of $10^{4.79}$ $TCID_{50}$ /mL was achieved and then inoculated to 8 L of bovine plasma for a final viral titer of $10^{3.76}$ $TCID_{50}$ /mL.

The UV-irradiated samples were PCR analyzed to evaluate the UV-C induced damage in the genome of PCV-2. DNA was extracted from irradiated plasma samples using the Nucleospin Virus Kit (Macherey-Nagel, Düren, Germany).

PCV-2 primers used to amplify a 1.3 Kb amplicon were: Forward 5'-ACATCGAGAAAGCGAAAGGA-3' and reverse primer 5'-ACCGCTGGAGAAGGAAAAAT-3'. Amplification was carried out

using an initial activation step at 94°C for 4 min, followed by 35 cycles at 94°C for 20 s, 60°C for 30 s and 72°C for 7 min; and a final step at 72°C for 7 min with a 4°C hold. A 5-μL sample of PCR product was analyzed by electrophoresis on a 1% agarose gel, in 1 × TAE (40 mM Tris-acetate, 1 mM EDTA). DNA was stained with ethidium bromide and visualized under UV light.

Real Time PCR of the same samples was carried out using the kit EXOone PCV2 (EXOPOL, Zaragoza, Spain).

6.2.7.2 PEDV

Eight liters of commercial liquid bovine plasma were inoculated with PEDV strain CV777 (Debouck and Pensaert, 1980) and irradiated at 0, 750, 1500, 3000, 6000 and 9000 J/L as previously described. PEDV was propagated in VERO cells (ATCC CCL-81) grown in Minimum Essential Medium Eagle (MEM-E; ThermoFisher, Waltham, MA, USA) supplemented with 10% FBS. Cells were cultured in 175-cm^2 flasks and when they were confluent, the media was removed, and cells were rinsed twice with PBS. Finally, inoculum was added at MOI 0.001 and adsorption was done for 1 hour at 37ºC. Subsequently, the inoculum was discarded, flasks were rinsed twice with PBS and MEM-E was supplemented with 1% penicillin 10,000 U/mL and streptomycin 10 mg/ml (ThermoFisher), 0.5% Nystatin (10,000 U/mL), 1% L-glutamine (200 mM), 0.05% trypsin and 0.3% tryptose. The viral stock was produced in the same cells to obtain 703 mL of suspension with a viral titer of $10^{5.42}$ $TCID_{50}$ /mL that was used to inoculate 8 L of bovine plasma achieving a final viral titer of $10^{4.7}$ $TCID_{50}$ /mL.

The UV-irradiated samples were analyzed by conventional PCR. RNA was extracted with Nucleospin Virus Kit (Macherey-Nagel, Düren, Germany). PEDV primers used to amplify a 1.48 Kb amplicon were: Forward 5’- ATTAATGGCGCTTGTGGTTC-3’ and reverse 5’-TAGCATTTCCTGCGCTTTTT-3’. Amplification conditions were set as follows: 94°C for 4 min, 35 cycles at 94°C for 20 s, 55°C for 30 s and 72°C for 7 min; and a final step at 72°C for 7 min with a 4°C hold. A 5-µL sample of PCR product was analyzed by electrophoresis on a 1% agarose gel, in 1 × TAE (40 mM Tris-acetate, 1 mM EDTA). cDNA was stained with ethidium bromide and visualized under UV light.

The same samples were analyzed by qRT-PCR using VetMAX™ PEDV/TGEV/SDCoV Kit (Thermo Fisher Scientific, Massachusetts, USA).

6.3 Results

6.3.1 Results of Bioassay

Among the 10 porcine plasma batches tested (Table 9), the one with highest PCV-2 load and lowest PCV-2 antibody values was selected (No. 9). This batch contained 2.71 x 10^4 PCV-2 DNA copies/mL (Ct = 30.96) and a Ct value of 35.35 for PRRSV European strain (no number of RNA copies were provided by the technique). Also, it had ELISA S/P ratios of 0.83 (low to moderate) for PCV-2 and 2.99 (high) for PRRSV antibodies. Furthermore, the selected plasma used as inoculum had antibodies against SIV, RVA, PPV, HEV and *S. enterica* and presence of low amounts of nucleic acid of SIV (Ct = 36.41), RVA (Ct = 37.6), PPV (Ct = 39.44) and HEV (Ct = 37.8) were detected (Table 10).

Table 9. Presence of antibodies and genome (copies/mL) of PCV-2 in different porcine plasma batches, including the selected one (No. 9).

Sample	Vol. sample	PCV-2 genome Copies/mL	S/P Elisa IgG PCV-2
1	100 mL plasma	1.12E+04	0.947
2	100 mL plasma	1.65E+04	0.864
3	100 mL plasma	2.06E+04	0.907
4	100 mL plasma	2.36E+04	0.898
5	100 mL plasma	2.06E+04	0.880
6	100 mL plasma	2.71E+04	0.891
7	100 mL plasma	1.76E+04	0.960
8	100 mL plasma	5.08E+03	1.011
9	**100 mL plasma**	**2.71E+04**	**0.834**
10	100 mL plasma	2.67E+04	0.863
Cut-off Negative			0.284
Cut-off Positive			0.334

Table 10. Presence of antibodies (Ab) and genome (PCR) of different infectious agents tested in the porcine plasma inoculum and in the different animal groups.

			Group 1		Group 2		Group 3		Group 4		Group 5	
	Plasma Inoculum		Negative control		Non-UV plasma		UV 3000 J/L plasma		UV 9000 J/L plasma		Positive control PCV-2	
Pathogen	Ab	PCR	Ab	PCR	Ab	PCR	Ab	PCR	Ab	PCR	Ab	PCR
PCV-2	+	+	-	-	-	-	-	-	-	-	+ (6/6)	+ (6/6)
PRRSV EU	+	+	-	-	+ (8/8)	+ (8/8)	-	-	-	-	-	-
PRRSV US	-	-	-	-	-	-	-	-	-	-	-	-
TGEV	-	-	-	na	-	na	-	na	-	na	-	na
SIV	+	+	-	na	-	na	-	na	-	na	-	na
PPV	+	-	-	-	+ (3/8)	Feces -	-	-	-	-	-	-
RVA	+	+	-	-	+ (1/8)	Feces -	-	-	-	-	-	-
PEDV	-	na	-	na	-	na	-	na	-	na	-	na
PRV	-	na	-	na	-	na	-	na	-	na	-	na
HEV	+	+	-	-	+ (1/8)	Bile -	-	-	-	-	-	-
SVDV	-	na	-	na	-	na	-	na	-	na	-	na
CSFV	-	na	-	na	-	na	-	na	-	na	-	na
BDV	-	na	-	na	-	na	-	na	-	na	-	na
S. enterica	+	-	-	na	-	na	-	na	-	na	-	na

Plasma inoculum (untreated abattoir collected porcine plasma used for bioassay); Group 1 (negative control group of pigs injected ip with saline); Group 2 (pigs injected ip with untreated abattoir plasma; 0 J/L); Group 3 (pigs injected ip with 3000 J/L UV-C treated plasma); Group 4 (pigs injected ip with 9000 J/L UV-C treated plasma); Group 5 (positive control group of pigs inoculated with PCV-2). Ab (serum antibody); PCR (serum genome); + (positive result); - (negative result); na (not analyzed); (n/n) number of pigs with positive antibodies or genome respect to the total number of pigs in the treatment; Feces – (negative fecal result); Bile – (negative bile result).

During the experimental period and at necropsy no clinical signs of infection were observed in any of the piglets.

The negative control group (group 1) remained free from PRRSV and PCV-2 and showed absence of seroconversion against those viruses during the entire study period. In contrast, all piglets from group 5 (positive control group, inoculated with PCV-2) became infected with PCV-2 within 15 dpi as measured by qRT-PCR. These pigs had already seroconverted against PCV-2 by the following sampling at 30 dpi.

All piglets in group 2 injected with raw native plasma that was not subjected to UV-C (0 J/L) were found PRRSV positive by qRT-PCR and seroconverted by 15 dpi. No evidence of PCV-2 infection by qRT-PCR or ELISA was detected in group 2 (0J/L UV-C) piglets. Three piglets of this group seroconverted against PPV at 50 dpi, without evidence of viral excretion in feces at 50 dpi. One piglet also seroconverted against RVA at 50 dpi, having a negative qRT-PCR in feces at 50 dpi and in sera at 15 and 30 dpi. By 50 dpi, one group 2 piglet (0 J/L) seroconverted against HEV without presence of virus in bile. No seroconversion against SIV was observed in group 2 (0 J/L) piglets.

Piglets in groups 3 and 4 injected intraperitoneally with UV-C irradiated plasma at 3000 J/L and 9000 J/L, respectively did not become viremic (qPCR or qRT-PCR) for PCV-2, PRRSV, SIV, PPV, RVA and HEV and they did not seroconvert at any time during the 50-dpi period.

6.3.2 Results of genome amplicon test vs qRT-PCR

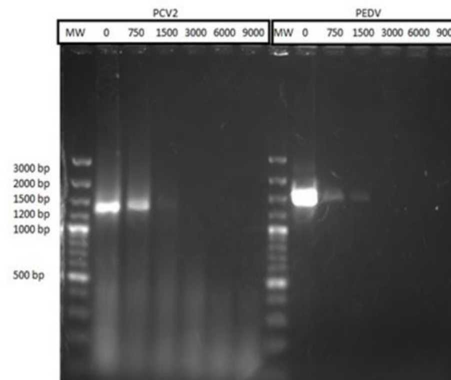
Regarding the effect of UV irradiation on the PCV-2 and PEDV genome, the viral amplicons were detected when the PCV-2 or PEDV inoculated bovine plasma was UV-C irradiated at 0, 750 and 1500 J/L, although as the subsequent irradiation doses increased to 1500 J/L, the PCR bands became weaker than that of the lower doses. At higher doses, 3000 J/L, 6000 J/L and 9000 J/L, the amplicon band was no longer observed (Figure 1).

The results of the PCV-2 and PEDV real time PCR test kits for the same inoculated bovine plasma samples are shown in the table 11, As can be observed, the Ct values were very similar for both viruses at the different UV irradiation doses, with very mild increases of 1.81 and 1.59 points between 0 to 9000 J/L for PCV-2 and PEDV respectively.

Table 11. PCV2 and PEDV Cq values analyzed by qPCR.

Sample Identification	Cq Mean	Sample Identification	Cq Mean
PCV2 Negative control	**Negative**	**PEDV Negative control**	**Negative**
PCV-2 0 J/L	16.3	**PEDV 0 J/L**	20.07
PCV-2 750 J/L	16.78	**PEDV 750 J/L**	20.37
PCV2 1500 J/L	16.82	**PEDV 1500 J/L**	20.53
PCV2 3000 J/L	17.05	**PEDV 3000 J/L**	20.53
PCV2 6000 J/L	17.82	**PEDV 6000 J/L**	21.04
PCV2 9000 J/L	18.11	**PEDV 9000 J/L**	21.66

Figure 12. PCV-2 and PEDV long amplicon PCR results. Beyond 1500 J/L no band was detected in both cases.



6.4 Discussion

The swine bioassay was used to demonstrate the effectiveness of the UV-C to reduce the viral load that may be present in liquid commercial plasma in an *in vivo* model. Intraperitoneal administration was selected as an administration route to avoid the possible inactivation or loss of viral particles due to the hydrochloric acid and digestive enzymes in the gastrointestinal tract (Kasorndorkbua *et al.*, 2002). By the intraperitoneal route, the main route of absorption of an inoculated material is through the mesenteric blood vessels, which drain into the portal vein and pass through the liver (Lukas *et al.*, 1971). In consequence, a certain amount of the injected liquid may pass directly across the diaphragm through small lacunae and into the thoracic lymph (Abu-Hijleh *et al.*, 1995) and then to systemic circulation. This technique has been shown to be a very sensitive model to detect infective pathogens (Gerber *et al.*, 2014; Dee *et al.*, 2015; Sampedro *et al.*, 2015).

Commercially collected liquid porcine plasma contained antibodies and presence of small amounts of nucleic acid of different viruses (PCV-2, PRRSV, SIV, RVA, PPV and HEV), which was consistent with endemic pathogens present in commercial swine population in the Catalonia, Spain region (de Deus *et al.*, 2007; López-Soria *et al.*, 2010).

All the piglets in group 2 injected intraperitoneally with 10 mL of untreated liquid porcine plasma (0 J/L) seroconverted against PRRSV and, a lesser percentage, against PPV, RVA and HEV. In the case of PPV, 3 piglets seroconverted against the virus but no excretion of PPV was found in feces at the end of the study or its detection in sera during the experimental period. Similarly, one piglet seroconverted to RVA while sera and feces remained PCR negative. Furthermore, one piglet seroconverted against HEV, while the bile remained PCR negative. This low seroconversion rate could be a result of the extremely low quantities of virus in commercially collected porcine plasma. However, pigs in groups 3 and 4 injected with liquid porcine plasma treated with UV-C (3000 J/L or 9000 J/L, respectively) did not seroconvert or become PCR positive demonstrating that the viral particles present in commercially collected porcine plasma were inactivated.

Interestingly, the commercially collected porcine plasma contained 10^4 DNA copies/mL PCV-2. However, piglets injected with untreated plasma (0 J/L) did not become viremic or seroconvert with PCV-2 indicating that the PCV-2 virus particles were not infective. The absence of PCV-2 infection could be either associated to the presence of neutralizing antibodies in commercial pooled plasma, an inadequate amount of PCV-2 in the intraperitoneal injection to infect the animals, or that the virus present in raw plasma was already inactivated despite a

positive PCR result. Neutralizing antibodies naturally present in pooled plasma were reported to inactivate up to 4 $\log_{10}$ $TCID_{50}$ of PCV-2 (Polo *et al.*, 2013). This observation also confirms that a positive PCR test does not imply that the pathogen is infective.

SIV RNA was detected in the raw plasma by qRT-PCR, but with a high Ct number. Since no piglets seroconverted to the virus and no presence of SIV was found in the analyzed samples (sera and lung qRT-PCR), it is very likely that the very low load of SIV present in the inoculum was unable to cause seroconversion. The low load of SIV present in the inoculum was likely a result of contamination at the abattoir. Due to the administration route selected, infection of the animals was not expected because SIV is an exclusively respiratory pathogen. However, if the amount of virus in the inoculum would have been greater, a seroconversion to the level of immunization could have happened.

The current data are consistent with previously published research demonstrating that viruses (PRV, PRRSV, SIV, PPV, PEDV, *Swine vesicular disease virus* (SVDV), CSFV, BVDV, SVA and PCV-2) added to bovine or porcine plasma are inactivated and, thus, not infective *in vitro* after UV-C treatment with the same Sure-Pure Turbulator used in the present study (Chapter 5).

The WHO (WHO, 2004) guidelines on viral inactivation and removal procedures intended to assure the viral safety of human blood plasma products establishes that a robust inactivation process should be capable of removing or inactivating at least 4 $\log_{10}$ of a wide range of viruses (WHO, 2004). In previous studies, the UV-C irradiation of liquid plasma was able to inactive 4 $\log_{10}$ of different bacteria and viruses (Polo *et al.*, 2015; Chapters 3, 4 and 5) and the present study also demonstrated

the lack of transmission of any virus after being UV irradiated at a minimum of 3000 J/L. Therefore, UV-C irradiation complies with the WHO definition of a robust inactivation process. Furthermore, the UV-C mechanism of inactivation (DNA or RNA damage) is different than the thermal inactivation provided by the spray-drying step.

Overall results indicate that liquid commercial plasma without any safety treatment and administered intraperitoneally to naïve susceptible pigs was infective for some viruses for which the initial plasma had virus genome. However, UV irradiation with the SurePure Turbulator system at 3000 J/L or more was enough to avoid transmission of any of the tested viruses to the animals. The irradiation at 3000 J/L was chosen as the level of irradiation for liquid plasma that is used commercially by a specialized blood plasma producer. The opaqueness of a liquid can limit the penetration of UV-C irradiation and therefore affect the efficiency of pathogen inactivation (Matak *et al.*, 2005; Groenewald *et al.*, 2013). However, if sufficient turbulence is introduced into the liquid, all material is exposed to the surface of the UV source therefore overcoming the limited penetration of the UV irradiation (Alberini *et al.*, 2015). The Sure Pure Turbulator (EP-1255444B1) is designed to create turbulent flow and has been successfully used to pasteurize opaque liquids like fruit juices, wine (Keyser *et al.*, 2008; Fredericks *et al.*, 2011) and milk (Donaghy *et al.*, 2009). In addition, in case of animal plasma, a UV dose of 3000 J/L has been demonstrated to have no effect on the functionality of proteins present in SDPP (Polo *et al.*, 2015; Cottingim *et al.*, 2017).

Increased UV dose (0, 750, 1500, 3000, 6000 or 9000 J/L) of PCV-2 and PEDV contaminated bovine plasma resulted in a progressive

loss of agarose gel bands, with no band detection beyond 1500 J/L. In contrast, the genome of both viruses is detected consistently in all samples by real time PCR. PCR assays of large amplicons, unlike Real-Time PCR using very short amplicons, can be used as a method to estimate the presence of potentially viable viral particles in the sample. These results demonstrated that a smaller amplicon that is typically used in diagnostic PCR tests may not detect the damage induced by UV irradiation. However, a larger amplicon, more likely to contain a sequence sensitive to UV irradiation, is more likely to detect a region that is damaged by UV irradiation. This further demonstrates that a positive qRT-PCR result does not demonstrate infectivity.

In summary, the results of this study demonstrate that UV-C treatment is a useful step to inactivate potential pathogen contamination of commercially collected animal plasma used to produce spray-dried animal plasma and the virus genome damage caused by UV-C irradiation may not be detected by commercial PCR tests using small amplicons.

Chapter 7

General discussion

Spray-dried plasma (SDP) is a functional protein source that, when included in pig nursery feeds, significantly improves daily gain, feed intake, production efficiency and reduces post weaning lag (Gatnau and Zimmerman, 1991; van der Peet-Schwering and Binnendijk, 1995; Cain and Zimmerman, 1997; Coffey and Cromwell, 2001; Van Dijk *et al.*, 2001; Torrallardona, 2010).

The manufacturing process of SDP involves several safety features as veterinary inspection at abattoir, pooling of plasma, spray-drying process and storage. Veterinary inspection is crucial to ensure that blood from only healthy animals slaughtered for human consumption is the exclusive source of raw material to be used for the manufacturing of blood products. However, early viremia/bacteremia and subclinical infections may not be detected in veterinary inspection. Therefore, trying to cope and counteract with this eventual scenario, it is vital to implement, know and validate the biosafety steps that are part of the manufacturing process of SDP, either inherently, such as the action of neutralizing antibodies, as well as the processes directly implemented to inactivate and/or eliminate pathogens.

The action of neutralizing antibodies has been demonstrated in various studies (Solheim and Seghatchian, 2006; Williams and Khan, 2010; Polo *et al.*, 2013) to be an inherent safety step that contribute to the safety of blood products. The spray-drying process conditions used during the manufacturing of SDP represent an additional step contributing to inactivate different pathogens (Polo *et al.*, 2002, 2005; Pujols *et al.*, 2007; Gerber *et al.*, 2014; Pujols and Segalés, 2014). Also, certain storage conditions (such as room temperature >20ºC for 14 days) have been demonstrate a safety treatment for some envelope viruses and

bacteria (Pujols & Segalés, 2014; Sampedro *et al.*, 2015;Annex 2). All these biosafety steps contribute to the global safety of the SDP, as it has been demonstrated in several *in vivo* studies (Opriessnig *et al.*, 2006, 2010, 2014, Pujols *et al.*, 2008, 2011, 2014; Patterson *et al.*, 2010; Shen *et al.*, 2011). Noteworthy, although the manufacturing process of the SDP consists of biosafety steps duly studied and validated, which effectiveness has been demonstrated in numerous studies, the security of the SDP is often questioned due to its nature as raw blood by-product, especially when emergent or re-emergent pathogens appear.

This PhD Thesis focused on the evaluation and validation of a new redundant pathogen inactivation step that may be implemented in the manufacturing process of the SDP: UV-C irradiation.

Finding a method of inactivation or removal of pathogens is not easy, since the nature of the product to be treated and how the product will be affected during the process must be taken into account. Moreover, the suitability of the method chosen within the industrial manufacturing process must be assessed.

Since SDP is produced from unfractionated plasma, which protein concentration is high, and the biological activity of these proteins must be preserved, the UV-C irradiation was profiled as a good candidate to be implemented in the manufacturing process as a new redundant biosafety step. Technological evolution has allowed the development of UV-C irradiation devices based on turbulent flow, which allow irradiating opaque fluids of high viscosity efficiently. In addition, these UV equipments have been widely used in the food industry for the treatment of other complex opaque liquids as milk or fruit juices.

UV-C irradiation is a great candidate to take into account in the face of the need to implement a redundant biosafety step, especially at industrial level in all those manufacturing working with large volumes of liquid or solid materials due to its ease of use and its ability to work with large volumes continuously. Despite requiring an initial investment, it is not an extremely expensive treatment and its maintenance is simple. It does not require the incorporation of chemicals on the initial product nor the withdrawal of these or their by-products, with which the processing of the product is minimal. Furthermore, this technology has shown in several studies carried out on different substances of therapeutic or alimentary interest, its ability to reduce the microbial load without practically altering the biological activity of the proteins, without meaningful oxidation of fats or modifying the organoleptic characteristics of treated products, such as water, juice, wine, vegetables or plasma derivatives. In the specific case of SDP, it was demonstrated that UV-C irradiation did not affect protein integrity and biological activity of plasma proteins, achieving *in vivo* the same results that plasma not treated by UV-C, without detrimental effects on pig performance (Polo *et al.*, 2015; Cottingim *et al.*, 2017).

Taking into account all above mentioned premises, the major aim of this PhD Thesis was to evaluate the effectiveness of a UV-C irradiation device based on turbulent flow, SurePure Turbulator™, when irradiating raw plasma artificially inoculated with various pathogens of interest in the swine industry and, when possible, their results were compared with those obtained by the spray-dry process in order to better characterize these two biosafety steps of the SDP production process.

In studies 1 and 2 (Chapters 3 and 4 and Annex 2), the UV-C effect on bacterial survival was assessed. In the study 1, *S. typhimurium*, *S. choleraesuis* and *E. faecium* where subjected to different UV-C doses. In the study 2, the survival of *Escherichia coli* (*E. coli*) K88 and K99 strains to UV-C and spray-dry treatments were analyzed. Both *Salmonella* spp. under study are considered important pathogens for pigs and, from a biosecurity point of view, they are two infectious agents to be taken into account since Regulation (EC) No. 142/2011 establishes that *Salmonella spp.* must be absent in 25 g of product. In the same regulation, total *Enterobacteriaceae* are regulated, being <300 cfu/g in 5 consecutive samples from de same production batch. Therefore, submitting these two *Salmonella* serovars and the two different *E. coli* strains to the same UV treatment allowed studying the response of different variants/strains to this safety treatment. Moreover, the effect of UV-C on *E. faecium* was analyzed in study 1. This bacterium has been described as a surrogate organism for *Salmonella spp*. in numerous thermal inactivation studies (Ma *et al.*, 2007; Kopit *et al.*, 2014) and, given its harmlessness, it can be used within the manufacturing facilities (California Almonds, 2014). These facts accounted for the reason to use this microorganism as a surrogate of *Salmonella spp*. in UV-C irradiation experiments.

In these two initial studies, the 4D reduction value was achieved at 2301 J/L for *S. choleraesuis*, 3186 J/L for *S. typhimurium*, 3364 J/L for *E. faecium*, 3105 J/L for *E. coli* K88 and 3427 J/L for *E. coli* K99. The 4D reduction value in all cases was very close to 3000 J/L, which is the dose applied under commercial manufacturing conditions. All bacteria showed non-linear inactivation kinetics, having special importance the cases of *S. typhimurium* and *E. coli* K88 and K99, in

which tails appeared in their inactivation kinetics curves. Moreover, the tail appeared in *S. typhimurium* discards the use of *E. faecium* as its surrogate. Tails appear in inactivation kinetics when, despite increasing the dose of UV-C, the reduction in the population slows down and is not proportional to the increase in UV-C irradiation. It is very important to take into account this reduction of the inactivation speed (inactivation constant, *k*) because, if it is not taken into account, the 4D reduction value would be lower, overestimating the inactivation capacity of the system (Geeraerd *et al.*, 2005).

Usually, the appearance of tails is related to microorganism aggregation, appearance of a resistant subpopulation, hydraulic design (Hijnen, 2006), matrix effect or particle size effect (Winward, 2008). Porcine plasma is a colored and viscous liquid matrix which characteristics may interfere with the UV-C irradiation process. It contains 8 to 10% solids, and a complex blend of different proteins with some proteins having binding properties (Burnouf, 2007). Therefore, the matrix and particle size effects of porcine plasma may have had a special impact on the tailing effects of the UV-C treatment.

When working in inactivation processes by UV-C irradiation, as the nucleic acid is the target of inactivation, one must take into account that microorganisms (especially unicellular ones) have DNA repair mechanisms capable of fully repairing them or mitigate the harmful effects caused by this radiation. In this PhD Thesis, a specific assessment has not been made in this regards since the inactivation stage by UV-C constitutes a first stage of inactivation. The plasma, once irradiated, is concentrated by reverse osmosis or nanofiltration and subjected immediately to the spray-drying process. The time that elapses between

one process and another is scarce, so, it is not likely that significant repair rates would be achieved. On the other hand, photoreactivation (a well-known mechanism of DNA repair) does not seem feasible under these conditions; given that all these industrial processes occur in closed circuits, in the absence of light

Salmonella spp. (Annex 2) and *E. coli K88* and *K99* subjected to spray-drying (under conditions close to the commercial manufacturing process, i.e., outlet temperature 80ºC and retention time 60 seconds) had log reduction factors of 5.30 and 5.35 for *S. choleraesuis* and *S. typhimurium* respectively, and more than 7 log in both *E. coli* strains. The UV treatment for the evaluated bacteria, showed log reduction factors very close or superior to the 4 log_{10} working in industrial manufacturing conditions. Taking into account the recommendations of WHO (2004), which were developed for the evaluation of inactivation/removal of viruses in human plasma derivatives, it can be determined that the spray-drying step had demonstrated their effectiveness in the inactivation of the studied bacteria.

Since UV-C and spray-drying process at 80ºC throughout its substance steps are based on different inactivation methods, they must be considered redundant biosafety steps, because their mechanism of action act on different targets. Since these redundant biosafety steps were applied to the same manufacturing process, the log reduction factors should be considered cumulative within the manufacturing process, thus increasing the overall clearance capacity of the system. In addition to this cumulative effect, a synergistic effect may occur, in which the overall reduction achieved would be greater than the arithmetic sum of the reduction factor obtained in each inactivation step (WHO, 2004).

In study 3 (Chapter 5), the effect of UV-C on different viruses of interest in the swine industry was analyzed, trying to choose viruses with different physical-chemical characteristics, types of genome (DNA or RNA), genome lengths (long and short genomes), presence or absence of envelope and resistance to inactivation processes. Also, viruses belonging to the same family and genus were selected to determine if they would have similar behaviors under UV-C irradiation. As stipulated by WHO (2004) guidelines for viruses infecting humans, it is always convenient to test the inactivation process with the virus of interest, choosing the strain with the greatest known resistance. However, it is also convenient to test viruses with different physical-chemical characteristics to obtain information about the robustness of the inactivation process (WHO, 2004).

The selection of enveloped viruses included PRV, PRRSV, PEDV, BVDV, SIV and CSFV and PPV, SVDV, PCV-2 and SVA were chosen as non-enveloped viruses. All these viruses were subjected to different UV-C doses and, by titration of the samples at each UV-C dose, the inactivation curve for each virus was constructed. In general terms, results showed that enveloped viruses have a higher sensitivity to UV-C than non-enveloped ones, being its 4D reduction value less than 2000 J/L in all cases. This may be due to intrinsic factors of the viruses, or to factors inherent to the irradiation process or to conditions in which the process is carried out. For example, it is known that UV-C irradiation generates reactive oxygen species (ROS) that may induce lipid peroxidation (Chan *et al.*, 2006; Phukan *et al.*, 2018), which could affect the lipid envelope. Also, the presence of anticoagulant in the plasma may help destabilizing the envelope or the most external layer of some viruses, as it has been previously shown (Estes and Kapikian, 2007).

Within the group of enveloped viruses, two representatives of the genus *Pestivirus* were studied: CSFV and BVDV. This allowed comparing their inactivation curves and assessing whether members of the same genus would react similarly to UV-C treatment and could be used as surrogate organisms. Study 3 indicated that both pestiviruses had similar inactivation kinetics with close 4D reduction values, being able to be used as surrogate organisms for each other. This observation may be of value to predict the expected inactivation behavior for other non-tested viruses of the same genus of some viruses studied in this project; however, it is always better to test the behavior of each virus of interest if technically feasible.

Regarding non-enveloped viruses, two types of inactivation behavior were recorded. On one hand, PPV and SVA have 4D reduction values very close to 3000 J/L (2161 J/L for PPV and 3223 for SVA). However, in the case of SVDV the 4D value increased to 3708 J/L, having found highly resistant viral particles in the blind passes of the 6000 and 9000 J/L doses. For PCV2, unfortunately, the 4D value was not able to be calculated for the impossibility to reach the 4 log_{10} $TCID_{50}$/mL in the inoculated plasma. Even so, its high resistance to treatment was demonstrated by inactivating only 2.28 log $TCID_{50}$/mL at 3000 J/L and 2.71 log at 9000 J/L. These results were not surprising because its well-known high resistance to inactivation treatments (Nims and Plavsic, 2012). PCV-2 and PPV, both a with small DNA genome size, are commonly compared due to their resistance to inactivation treatments, especially the thermal ones (Nims and Plavsic, 2012). However, when subjected to UV-C treatment they had very different log reduction factors and 4D values. This may be due to differences in their genome: PPV presents a 5.7 kb linear ssDNA genome while PCV2 has

a circular ssDNA genome of 1.77 kb. PCV-2, therefore, has a circular genome 3 times smaller than PPV, which could confer greater resistance to UV-C. In addition, the lower the genome, the lower the target nucleotides to be altered by UV-C, which can explain the results observed with these two small viruses.

Regarding the influence of the composition and size of the genome on the effectiveness of UV-C, it has been described that viruses with DNA genomes are more susceptible to UV-C (Rauth, 1965; Kowalski, 2009) and that the larger their genome, the greater the susceptibility (Lytle and Sagripanti, 2005). These statements have been demonstrated in some cases (Wang *et al.*, 2004), but in the present PhD Thesis no correlation between these parameters was found. Noteworthy, the number of analyzed viruses in this project is limited and whatever generalization regarding susceptibility and genome length might be too speculative.

While analyzing the kinetic models for each studied virus, appearance of biphasic kinetics was frequently found, as has been described by EMEA, ICHQ5A and WHO (EMEA, 1996; ICH, 1999; WHO, 2004). In addition, shoulders and tails frequently appear in inactivation kinetics, phenomena that can be attributed to the hydrodynamic design of the UV-C device (Hijnen, 2006), the presence of resistant viral subpopulations (Hijnen, 2006), the aggregation of viral particles with cell debris or debris (Cutler *et al.*, 2011), the aggregation of viral particles with each other or the plasma matrix and particle size effect (Winward, 2008). The present Thesis cannot rule out aggregation of viral particles, but studies were carried out using non-concentrated stock viral solutions, which are considered conditions to avoid such

aggregation. Plasma matrix seems to be the most determining factor, as can be deduced when comparing obtained data with other available UV-C irradiation studies. To effectively irradiate the plasma and achieve a 4D reduction value, it is necessary to use much higher doses of UV-C (3000 J/L = 2998.7 mJ/cm^2) than those used in water, over 200 mJ/cm^2 (Hijnen, 2006) or PBS or cell culture medium, under 200 mJ/cm^2 (Meunier *et al.*, 2017). However, the degree of UV irradiation level found in animal plasma is at the same range of irradiation than other opaque liquids commonly used in the food industry, as in the treatment of juices and wine, that uses UV-C irradiation doses between 2000 and 3000 J/L (Fredericks *et al.*, 2011), milk , over 1800 J/L (Donaghy *et al.*, 2009), or tea, irradiated at 3000 J/L (Monyethabeng and Krügel, 2016).

The overall results of the use of UV-C irradiation on viruses demonstrated that it is a useful technology to significantly reduce the plasma viral load. Taking into account that the UV-C is applied as a redundant biosafety step before the spray-drying process, the reduction factor achieved in this step would be added to that obtained by this latter process, whose effectiveness has been demonstrated previously for some of these viruses such as PRSSV (Polo *et al.*, 2005), PRV (Polo *et al.*, 2005), PCV-2 (Shen *et al.*, 2011), SVDV (Pujols *et al.*, 2007) and PEDV (Gerber *et al.*, 2014; Pujols and Segalés, 2014).

To validate the effectiveness of the plasma UV-C irradiation measured by means of viral load reduction in cell culture, a bioassay was carried out with different groups of piglets inoculated intraperitoneally with UV-C irradiated plasma at 0 J/L (untreated plasma), 3000 J / L, and at 9000J /L (Chapter 6). The results of the bioassay showed that none of the pigs in the groups that received the plasma irradiated by UV-C were

infected or seroconverted against the viruses which genome was detected in the initial plasma (PCV-2, PRRSV (European strains), SIV, PPV, HEV, RVA), thus confirming the efficacy of UV-C demonstrated in vitro in the previous study. It is important to state that detection of a viral genome in the raw plasma does not imply infectivity by a given virus, so, the swine bioassay was the ultimate approach to ascertain the infectiousness of the detected viruses or genome of these viruses in the plasma material.

In the group of pigs that received the non-irradiated plasma, all pigs were found viremic and seroconverted against PRRSV, three seroconverted against PPV, one to RVA and another to HEV. None of these pigs displayed clinical signs or lesions appreciable at necropsy and, furthermore, PPV, RVA and HEV were not found in serum, feces or bile (in the case of HEV), probably because the amount of virus present in the initial sample was so extremely low that these viruses were unable to produce consistent infections. On the other hand, although the commercially collected porcine plasma contained 10^4 DNA copies/mL PCV-2, piglets injected with untreated plasma (0 J/L) did not seroconvert to PCV-2 indicating that these PCV-2 genome copies did not corresponded to enough amount of viable infective virus or, alternatively, virus particles would have been neutralized by the action of neutralizing antibodies (Polo *et al.*, 2013).

Despite the implementation of UV-C and spray-drying as inactivation processes has shown its effectiveness in reducing bacterial and viral loads in plasma, the presence of some of these pathogen genomes (especially viruses) detected in the final product by real time PCR (rt-qPCR) generates doubts about its innocuity. The biosafety steps

applied on plasma consist of methods that even if they manage to inactivate the pathogens, they do not eliminate them from the product, remaining traces of genetic material detectable by rt-qPCR. However, this technique is not able to differentiate between viable and not viable viral particles (WHO, 2004). To demonstrate that UV-C is actually capable of inactivating viruses by generating damage in their genome resulting in non-infective viral particles, a conventional long amplicon PCR was designed to amplify fragments of approximately 1.7 kb of the genomes of PCV-2 and PEDV. The results of these PCR methods indicated that, as the UV-C irradiation dose increased, the genetic material probably accumulated damages that prevented the correct progression of the polymerase. In consequence, the higher the dose of UV-C, the lower the potential (if any) number of copies of that fragment that can be synthesized by the polymerase (Wellinger and Thoma, 1996). However, this loss of genome integrity was only observable using PCR methods yielding long amplicons; in contrast, same samples analyzed by rt-qPCR (amplifying short viral sequences), stable Ct values were always obtained. In conclusion, rt-qPCR methods are not able to differentiate the effect of the treatment on the integrity of the genome of the viral particles.

Overall results of the present PhD Thesis showed that the SurePure™ UV-C Turbulator design was effective in inactivating a wide range of bacteria and viruses spiked and naturally present in commercially collected liquid animal plasma. Since the UV-C mechanism of inactivation targets de nucleic acids and it is different than the thermal inactivation used in the spray-drying process, the UV-C can be considered an independent biosafety step in the manufacturing process of SDP. Moreover, such step would comply with the general

recommendations for the design of redundant biosecurity steps in the productive processes of blood products. Furthermore, this technology can be easily incorporated into the manufacturing process, and in fact is used already in some spray-drying industrial plants in USA. In conclusion, UV-C irradiation of liquid plasma is an apparently suitable additional inactivation step for the industrial production process of SDP.

Chapter 8

Conclusions

1. All tested bacteria, *Salmonella typhimurium, Salmonella choleraesuis, Enterococcus faecium, Escherichia coli K88* and *Escherichia coli K99* were sensitive to UV-C treatment, achieving 4 log_{10} reduction for all bacteria with an irradiation dose around 3000 J/L.

2. A 4 log_{10} titer reduction of all enveloped viruses tested (*Pseudorabies virus*, *Porcine reproductive and respiratory syndrome virus*, *Porcine epidemic diarrhea virus*, *Bovine viral diarrhea virus*, *Classical swine fever virus* and *Swine influenza virus*) was achieved at 2000 J/L or lower doses, with similar 4D values.

3. Non-enveloped viruses analyzed (*Porcine parvovirus, Swine vesicular disease virus, Porcine circovirus 2* and *Senecavirus A*) had higher 4D values than enveloped viruses. Two types of behavior were observed with non-enveloped viruses: PPV and SVA showed 4D values around 3000 J/L while SVDV had the highest 4D value. The 4D value was not able to be calculated for PCV-2, which was partially resistant to UV-C achieving a log_{10} reduction factor of 2.71 log.

4. Native porcine plasma PCR/RT-PCR positive to PRRSV, PCV-2, SIV, PPV, RVA and HEV irradiated at 3000 J/L and 9000 J/L did not transmit these viruses when inoculated intraperitoneally to naïve pigs. Only native, non-treated plasma caused seroconversion for several pathogens (PRRSV, PPV, RVA and HEV) in inoculated pigs.

5. UV-C treatment developed by SurePure with turbulent flow was useful to significantly decrease the porcine pathogen load in commercially collected liquid animal plasma. UV-C irradiation can be potentially included as an additional redundant virus and bacteria inactivation step in the industrial manufacturing process of spray-dried animal plasma.

Annex 1

SurePure Turbulator^{TM}

A1.1 SurePure Turbulator^{TM} SP1: pilot scale UV-C system

The UV-C reactor system (SP1, Fig.13 A) was designed and manufactured by Sure Pure Operation AG (Zug, Switzerland), and consists of a closed system with one low pressure mercury UV lamp (30 UVC Watts, 254 nm; Fig .13 Diagram B) surrounded by a quartz crystal which avoids contact with the product. The plasma flows through a steel tube containing a vortex (internal striped spiral tube), which generates a turbulent flow. The liquid flows between the corrugated spiral tube and the quartz sleeve. The tangential inlet of the reactor creates a high velocity and turbulence in the inlet chamber and brings the liquid into contact with the UV-C radiation. The liquid is pumped from the inlet chamber into the reactor (Fig. 13 Diagram B), through the gap between the quartz sleeve and the corrugated spiral tubing, at a minimum flow rate of 3800 L/h with a Reynolds value in excess of 7500, indicating turbulent flow. Plasma was recirculated by the pump from the tank to the UV-C lamp and recirculated many times through this circuit to achieve the required UV-C dose versus time. Liquid flow was controlled by a flow meter (Fig. 13 Diagram B). Flow rate was adjusted to 4000 L/h. The time spent by the liquid (8L total volume) to pass through the system once was 7.2 s, delivering 22.95 J/L or 22.94 mJ/cm^2 per cycle.

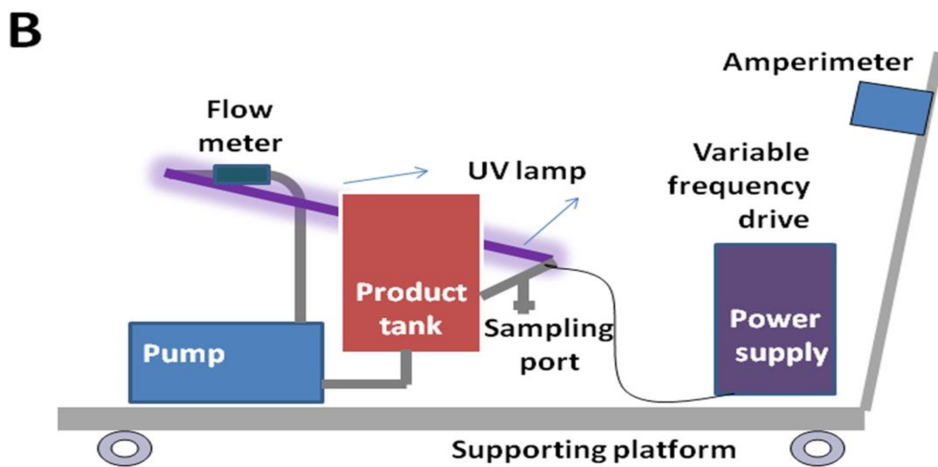


Fig. 13. A. Picture of the UV system SP1 used in the experiments. B. Diagram of the different elements of the system.

The establishment of the applied dose was performed according to the specifications of the machine given by the manufacturer (SurePure). Since the UV light was used initiallyto disinfect surfaces, irradiance is expressed as Watts per square centimiters (W/cm^2). UV dosage is the

product of time multiplied by irradiance, so W·s/area of the treatment device (Matak *et al.*, 2005). For liquids, UV dosage is expressed as J/L according to Keyser *et al.*, 2018.

A1.2 Calculation of the UV dosage per area

The length of the quartz cylinder used was 0.860 m with an outer surface area of 661.93 cm^2.

The space existing between the quartz sleeve and the corrugated spiral tubing is termed the annulus and the volume thereof was determined as being 0.675 L. The effective area (As) of UV-C was at a distance of 5 mm, since the lamp was located 5 mm away from the outer surface of the sleeve.

According to the manufacturer of the SP1, the energy transmission rate (total UV-C output) to the constant surface of the quartz sleeve (As = 661.93 cm^2) from the UV lamp was 25.5W (watts) UV-C. Disregarding the volume of the annulus and the type of product in the annulus, the following calculations were based on the effective As of the quartz sleeve alone.

The intensity (I) per reactor was calculated as follows:

Intensity (I) = Total UV-C output per unit (W) / Area (cm^2) = 25.5 W / 661.93cm^2=0.039 W/ cm^2= 38.5 mW/ cm^2.

The retention time (T) of the product per reactor was calculated as follows:

Retention time (T) = Volume of the reactor (l) / Flow rate L/h= 0:662 L/4000 L/ h = 0:662 L/1:111 L /s = 0:596s.

Thus, at a flow rate (Fr) of 4,000 L/h, the product retention time (T) was 0.596 s per reactor; therefore, the UV dosage (D) per surface area for one reactor with continuous flow was calculated as follows:

Dosage = Intensity (I) x Time (T) = 38.50 mW/cm^2 x 0.596 s = 22.94 mWs/cm^2 = 22.94 mJ/cm^2.

A1.3 Calculation of the UV dosage per volume

At a flow rate of 4,000 L/h, the T was 0.596 s per reactor, therefore the UV dosage per L of liquid treated for one reactor with continuous flow was calculated as follows:

Dosage = Total UV-C output per unit (W) / Flow rate (L/s) = 25.50 W / 1.11 L/s = (25.50 J/s)/ (1.11 L /s) = 22.95 J/L.

The industrial SurePure Turbulator™ device consists of 360 UV-C lamps that operate simultaneously. This industrial equipment consists of a radiometer to verify that the equipment operates at the selected UV-C dose. Unfortunately, in this pilot equipment (SP1), it was not possible to install the radiometer due to technical issues. Even so, when we performed the set-up of the equipment prior to the experiments, we did check, by using an ammeter, that the input current to the machine and its consumption were appropriate according to the technical specifications and the data sheet of the UV-C lamp provided by SurePure.

Annex 2

Combined effects of spray-drying conditions and post-drying storage time and temperature on *Salmonella choleraesuis* and *Salmonella typhimurium* survival when inoculated in liquid porcine plasma

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A2.1 Introduction

Spray-dried blood products (SDBP) are used in human food and animal feed. Ingredients like spray-dried plasma (SDP) or spray-dried red blood cells are used in the food and meat industry to provide texture, emulsion capacity and natural color properties (Appiah and Peggy, 2012). Likewise, SDP is an ingredient extensively used globally in pig feed due to its well-known beneficial effects on post-weaning performance and survival (Torrallardona, 2010). In contrast, pathogen contamination of animal-based ingredients is a major safety concern for both food and feed industries.

Spray-drying is based on the desiccation of a solution or suspension into a dried particulate form by spraying the feed into a hot drying chamber. The spray-drying process involves four stages of operation: (1) atomization of liquid source into a hot chamber; (2) contact between the spray and the drying medium (very hot air at a high gas mass to liquid mass flow volume ratio); (3) moisture evaporation resulting in particle formation; and (4) separation of dried products from the air stream (Kuriakose and Anandharamakrishnan, 2010). During the spray-drying process, computer systems designed to control and monitor processing temperatures and conditions are used to ensure that SDBP have been exposed to a minimum of 80ºC throughout its substance. This is one of the most important critical control points in the manufacturing process of SDBP intended for human or animal consumption.

Also, SDBP have low moisture (<9%) and very low water activity (a_w<0.6). Some pathogens, especially bacteria and enveloped viruses, are not able to survive for a prolonged time in dried materials like SDBP (Perdana *et al.*, 2013; Sampedro *et al.*, 2015; Pérez-Bosque

et al., 2016). Therefore, most SDBP manufacturers have adopted post-processing storage of SDP of porcine origin at room temperature (>20°C) for at least two weeks after production as an additional safety feature (Sampedro *et al.*, 2015). Thus, the sequential action of spray-drying and storage at room temperature for at least two weeks after spray-drying and packaging is able to inactivate microorganisms.

Salmonella enterica subsp. enterica Serovar Typhimurium (*S. typhimurium*) is a cause of acute foodborne zoonosis worldwide (Hohmann, 2001) and pigs are important reservoirs (Gebreyes *et al.*, 2004). *S. typhimurium* is the second most common serotype associated with foodborne illness. *Salmonella enterica subsp. enterica Serovar Choleraesuis* (*S. choleraesuis*) is frequently reported in North America and Asia (Gray *et al.*, 1995; Boyen *et al.*, 2008) as causing disease in pigs, with a lower prevalence reported in Europe. *S. choleraesuis* has also been described as able to cause systemic infections in humans (Chiu *et al.*, 2004).

The objective of this study was to determine the effectiveness of the spray-drying process on the inactivation of *S. choleraesuis* and *S. typhimurium* spiked in liquid porcine plasma. In addition, a second objective was to test the additive effect of immediate post-drying storage of the dried samples at two different storage temperatures 4.0 ± 3.0ºC or 23.0 ± 0.3ºC (room temperature) for 15 days on the inactivation of both *Salmonella* strains.

A2.2 Materials and methods

A2.2.1 Bacterial strains and test products

S. choleraesuis (ref.: UMI-UAB 46429) and *S. typhimurium* (ref.: UMI-UAB 46450) strains were provided by the UMI-UAB (Veterinary School, Infectious Diseases Unit, Universitat Autònoma de Barcelona, Spain). Inocula of both *Salmonella* spp. strains were prepared separately, growing one colony of each bacterium in TSA plates. After 24 hours of growth at 37ºC, bacteria were collected with a Kolle handle and re-suspended in 10 mL PBS.

Commercial spray-dried porcine plasma (SDPP; AP820P Lot # Y630962-357, APC Europe S.L., Granollers, Spain) was sterilized by γ-cobalt-60 irradiation at 10 kGy (Aragogamma S.A., Les Franqueses del Vallés, Barcelona, Spain) to eliminate any potential bacterial contamination. The γ-irradiated SDPP was diluted 1/10 in sterile distilled water (0.6 kg SDPP + 6 kg of water) to obtain 6.6 kg of liquid plasma containing around 8.5% solids. After solubilization, liquid plasma was passed through a sterile tissue to eliminate any insoluble material. Three 2 kg aliquots were obtained from the 6.6 kg of diluted plasma for spray-drying. A 10-mL inoculum of each bacterium containing around 10^{10}-10^{12} CFU/mL was prepared and used to inoculate each 2.0 kg aliquot of plasma to achieve a minimum final titer of approximately 10^{8}-10^{10} CFU/mL. This procedure was conducted in triplicate for each bacterium and was done in a sterile biological safety cabinet to avoid external contamination.

A2.2.2 Spray-drying test

Two kg of re-suspended SDPP (8.60 ± 0.01% solids) were inoculated with 10 mL of either the *S. choleraesuis* or the *S. typhimurium* isolates. From each aliquot of 2 kg of inoculated plasma, two bottles of 1 kg were obtained and one bottle was spray-dried at an inlet temperature of 200 ± 5ºC and an outlet temperature of 80 ± 1ºC and the other 1 kg bottle was spray-dried at the same inlet temperature with an outlet temperature of 71 ± 1ºC. Before drying the inoculated plasma, the spray-dryer was stabilized with water followed by non-inoculated plasma to achieve the combination of inlet and outlet temperatures of interest (Büchi Mini Spray Dryer B-290, Büchi Labortechnik, Switzerland). All inlet and outlet temperature combinations were performed in triplicate. Air flow through the column was set at 20-27 m^3/h at 20ºC. Estimated dwell time was <1 second. Before spray-drying, each inoculated bottle was sampled for bacterial plate count and solids analysis.

Once SDPP was obtained at the two designated outlet temperatures, each dried spiked sample was distributed in 27 glass tubes (0.5 cm length; inner diameter of 8 mm) containing 0.5 g of product. Three tubes were immediately seeded on TSA plates for bacterial count. Three samples were immediately stored at room temperature (23.0 ± 0.3ºC) and 3 more samples were immediately stored at refrigerated temperature (4.0 ± 3.0ºC) for 15 days, and then analyzed for bacterial count.

Particle residence time (RT) in a laboratory spray-drier is typically <1 second and particles cool very rapidly, whereas commercial driers have a RT ranging from 30 to 60 seconds thus particles have an extended time of heat exposure. To simulate the longer RT typical of

commercial driers, 9 dried samples kept in sealed glass tubes were placed in a water bath set at 89-91ºC for a RT of 30 seconds (actual temperature of the powder sample was 70.4ºC) and 9 more samples were held for a RT of 60 seconds (actual temperature of the powder sample was 80.7ºC). Upon completion of each RT, three tubes of samples held at either 30 or 60 seconds RT were stored at room (23.0 ± 0.3 ºC) or refrigerated (4.0 ± 3.0 ºC) temperature for 15 days before being analyzed for bacterial count. The study design is summarized in Fig. 14. These procedures were conducted in triplicate for each *Salmonella* strain.

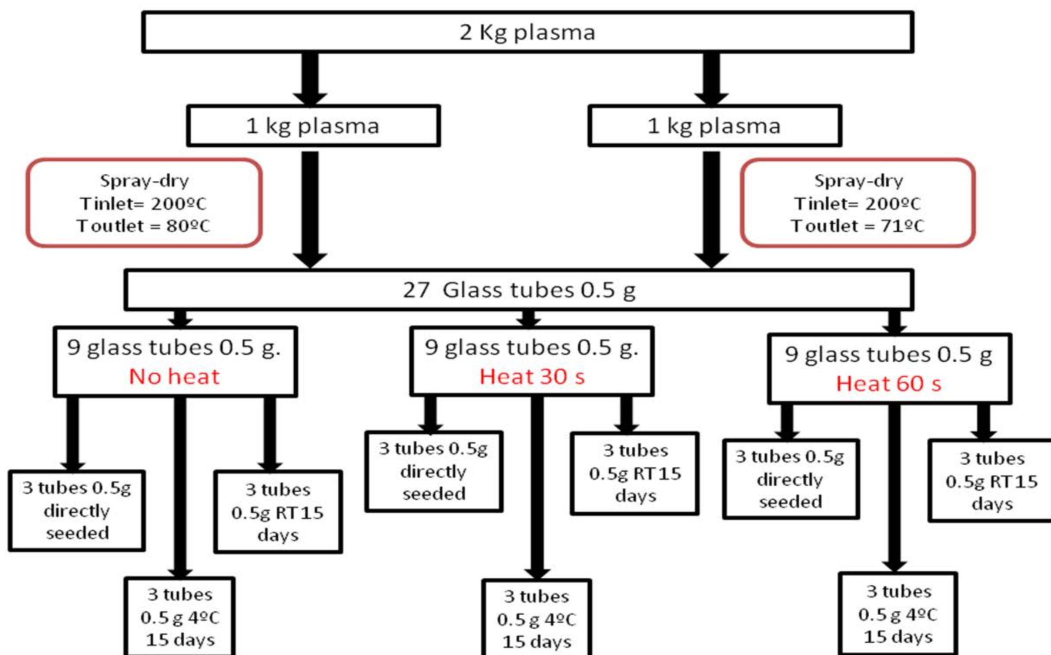

Fig. 14. Experimental design of the study. One kg of plasma was spray-dried at an outlet temperature of 80ºC ±1ºC and another kg was spray-dried at an outlet temperature of 71ºC ±1ºC. The same procedure was conducted in triplicate. In addition, each tube was analyzed in triplicate. RT: Room Temperature.

Bacterial count was done on TSA plates in triplicate for both liquid and dried samples. Each tube containing 0.5 g of dried sample was re-suspended in sterile water at 1:9 ratio. From this resuspension, 0.1 mL was seeded in TSA agar for 24 h at 37ºC. The colony counts were done following the ISO 7218:2007 guidelines. Results were expressed as a log_{10}/g of solids using the equation: log_{10}/g = log_{10} (CFU/mL / [(% solid content of re-suspended sample) / 100].

Liquid inoculated plasma samples were analyzed immediately after inoculation but also during an 8 h-period after inoculation to determine if the liquid plasma had an effect on reducing *Salmonella spp.* survival independently of the spray-drying effect. The liquid samples were maintained at 4.0 ± 3.0 ºC during this period and analyzed every 2 hours.

Liquid and spray-dried samples were analyzed for dry matter (AOAC method 925.45) to allow expression of the microbial inactivation results by grams of solids.

A2.2.3 Statistical analysis

Data were expressed by means of Log_{10} values and standard deviations of three independent experimental batches. Experimental data were analyzed as a 2 x 3 factorial arrangement of treatments using PROC GLM of SAS (SAS Institute. Cary, NC). Independent factors were outlet temperature (80 vs 71ºC) and residence time (0, 30 or 60 seconds). Least

square means were reported and differences at *P*<0.05 were considered significant.

A2.3 Results

All samples before *Salmonella spp.* inoculation showed an initial total plate count <10 CFU/mL, which was the limit of detection.

Plasma inoculated with *S. choleraesuis* strain had an initial count of 10.12 ± 0.17 log_{10}/mL and the average count of plasma inoculated with *S. typhimurium* was 9.56 ± 0.17 log_{10}/mL (Table 11).

Plasma inoculated with *S. choleraesuis* and spray-dried at inlet temperature of 200 ± 5ºC and the two-outlet temperatures indicated reduction of bacterial counts as shown in Table 11. A higher reduction of *S. choleraesuis* at 80ºC outlet temperature was observed, although it was not statistically different ($P = 0.510$) from 71ºC. The effect of RT presented a log polynomic regression inactivation curve with an R^2 of 0.99 (Figures 15A and 15B) for both outlet temperatures. Higher reduction ($P < 0.001$) was observed with prolonged RT. In addition, storage of all dried samples at either 4.0 ± 3.0ºC or room temperature (23.0 ± 0.3ºC) for 15 days eliminated surviving *S. choleraesuis* in dried plasma regardless of the spray-drying conditions or RT. When liquid plasma was inoculated with *S. choleraesuis,* stored in refrigerated temperature (4.0 ± 3.0ºC), and seeded in TSA every two hours for an 8-hour period post-inoculum, the bacterial count was maintained at an average of 9.11 ± 0.05 cfu log_{10}/mL almost without variation during the entire 8-hour period.

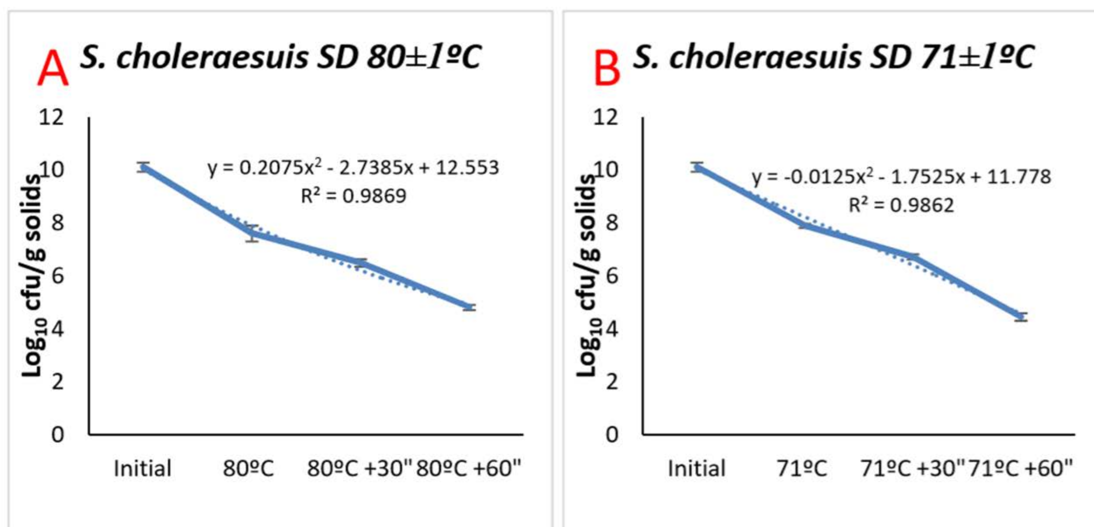

Fig. 15. ***S. choleraesuis*** **viability in SDPP samples produced at an outlet temperature of 80±1ºC (A) or 71±1ºC (B) and held at different residence times.**

Dotted line provides the exact data obtained in the experiment. Solid line is the calculated linear regression from the data obtained.

S. typhimurium inoculated in plasma and spray-dried at inlet temperature of 200ºC ± 5ºC and outlet temperature of 80±1ºC or 71 ± 1ºC had a reduction of bacterial counts as shown in Table 11. A significant higher (P <0.001) reduction of *S. typhimurium* for 80ºC outlet temperature was found compared to 71ºC. The inactivation kinetics presented a polynomic regression curve with an R^2=0.97 (Figures 16A and 16B) for both outlet temperatures when the RT was applied. Also, a higher reduction of *S. typhimurium* with prolonged RT (P < 0.001) was observed. When liquid plasma was inoculated with *S. typhimurium* and seeded in TSA plates, no significant changes in counts (8.69 ± 0.12 cfu

log_{10}/mL) over an 8-hour period were detected. Furthermore, as observed with *S. choleraesuis*, storage of all dried samples at either 4.0 ± 3.0ºC or room temperature (23.0 ± 0.3ºC) for 15 days eliminated surviving *S. typhimurium* in dried plasma independently of the spray-drying conditions or RT.

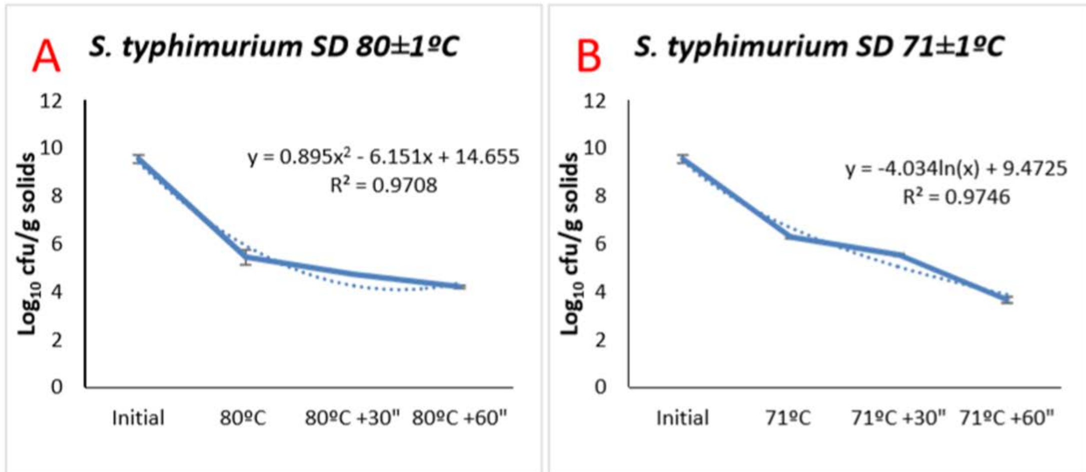

Fig. 16. *S. typhimurium* viability in SDPP samples produced at an outlet temperature of 80±1ºC (A) or 71±1ºC (B) and held at different residence times.

Dotted line provides the exact data obtained in the experiment. Solid line is the calculated linear regression from the data obtained.

Table 11. Effect of spray-drying porcine plasma at 200ºC±5ºC inlet temperature and two different outlet temperatures (80±1 and 71±1ºC), and kept at 3 different residence times (0, 30 or 60 seconds) on the inactivation of *Salmonella choleraesuis* and *Salmonella typhimurium*.

	S. choleraesuis CFU **Log_{10}/g solids**	***RF***	***S. typhimurium*** **CFU Log_{10}/g solids**	***RF***
Inoculated Plasma	10.12±0.17		9.56±0.17	
71ºC SDPP at 0s RT	7.90±0.08	-2.22	6.29±0.06	-3.27
71ºC SDPP at 30s RT	6.73±0.1	-3.29	5.55±0.06	-4.01
71ºC SDPP at 60s RT	4.46±0.14	-5.66	3.67±0.13	-5.89
80ºC SDPP at 0s RT	7.61±0.3	-2.41	5.45±0.31	-4.11
80ºC SDPP at 30s RT	6.50±0.14	-3.62	4.74±0.02	-4.82
80ºC SDPP at 60s RT	4.82±0.1	-5.3	4.21±0.06	-5.35
Statistical analysis				
SEM	0.09	0.08		
Temp	0.51	< 0.001		
Time	< 0.001	< 0.001		
Temp*time	0.009	< 0.001		

SDPP: liquid porcine plasma spray-dried at 200ºC inlet temperature and either 71ºC or 80ºC outlet temperature; RT: residence time of post-heating treatment after spray-dry of 30s (70.4ºC) and 60s (80.7ºC); RF: Log10 Reduction Factor; SEM: standard error of the least square means; Temp: main effect of outlet temperature; Time: main effect of residence time; Temp * time: interaction of effects of Temp and Time.

The present study showed that both spray-drying conditions and extended RT post-drying had a significant effect on reducing the survival of both *Salmonella spp.* strains studied. In addition, storage time at either 4.0 ± 3.0 ºC or 23.0 ± 0.3 ºC for at least 15 d was shown to eliminate the remaining detectable viable bacteria.

A2.4 Discussion

During spray-air contact, droplets interact with the hot air in the spraying chamber. Initially, as moisture is lost, the particle is maintained at the adiabatic wet bulb temperature, then, the droplet temperature increases to reach a value close or similar to the outlet air temperature (Straatsma *et al.*, 2007; Perdana *et al.*, 2013, 2015). Inlet and outlet temperature are the two main parameters that have a major influence on the inactivation of microorganisms. Inactivation occurs predominantly during the initial period of drying, while the remaining drying time further decreases moisture content (Perdana *et al.*, 2013, 2015). The survival of microorganisms is reduced by increasing the inlet temperature, but the outlet air temperature has the greatest impact on pathogen inactivation because this is the minimum temperature that the

particle will achieve during the drying process; therefore, higher outlet temperature typically results in higher microbial inactivation (Perdana *et al.*, 2013, 2015). Relatively high drying temperatures and rapid dehydration are two phenomena involved in microbial inactivation. Although the most important site of damage caused by dehydration is the cytoplasmic membrane (Crowe *et al.*, 1987; Lievense and Van't Riet, 1994), dehydration also produces damage to DNA/RNA and proteins (Lievense *et al.*, 1992). Results of the current study demonstrated that a greater reduction for both *Salmonella spp.* counts was observed at the higher outlet temperature, although it was only statistically significant for *S. typhimurium*. The minimum outlet spray-drying temperature is 80ºC for the commercial manufacturing process of SDP (Pérez-Bosque *et al.*, 2016) and results of the present study suggested that both *Salmonella spp.* strains were susceptible to spray-drying even at a lower outlet temperature (71°C).

Laboratory spray-dryers are useful for establishing guidelines to scale up the industrial production of SDP. The main differences between laboratory and pilot plant dryers compared to industrial dryers are design, size and volume processed, all of which affect the retention or dwell time of the product within the chamber (Foster and Leatherman, 1995). Laboratory spray-dryers have reduced retention or dwell time of the product within the chamber (<1 sec) compared with commercial driers (between 20 and 90 seconds, depending upon scale and design of the dryer). Furthermore, there is an immediate cooling to room temperature of the small quantity of dried product produced by lab-dryers in comparison with industrial dryers which process much larger quantity of material that extends the time for dissipation of heat from the dried product. Present results indicated that when extended RT was

simulated after drying liquid plasma with a lab-dryer at temperatures around 71 or 80ºC, there was a significant reduction on survival of both *Salmonella spp.* strains that was directly related with the higher RT regardless of outlet drying temperature. These results may confirm that commercial driers may be more effective than lab driers to inactivate microorganisms as suggested by Perdana et al. (2013).

Furthermore, SDBP are dry products with low moisture (<9%) and very low water activity (a_w <0.6). Some pathogens, especially bacteria and enveloped viruses, are not able to survive for prolonged periods of time in dry materials like SDBP (Sampedro *et al.*, 2015). Several mechanisms affecting microbial survival in dry materials have been described, such as, oxidative stress and reactive oxygen species formation which produces lipid peroxidation, and the browning reaction of sugars that cause protein denaturation and DNA damage. These changes are accumulative and have lethal effects on bacterial metabolism (Hernández-García, 2011). Therefore, as an additional safety feature, most manufacturers package and store porcine SDBP at room temperature (>20°C) for at least 14 days before release for sale. These storage conditions have been demonstrated as effective to inactivate certain pathogens susceptible to dry environments and mild temperatures, such as PRRSV, PEDV and coronaviruses in general (Pujols and Segalés, 2014; Sampedro *et al.*, 2015). The present study showed that *S. choleraesuis* and *S. typhimurium* did not survive in dried samples of plasma stored for 15 days after production at 4.0 ± 3.0 ºC or 23.0 ± 0.3 ºC, regardless of the outlet temperature used during drying or the post-drying residence time.

Under the conditions of this study, the combinations of spray-drying and RT followed by post-drying storage at 4.0 ± 3.0 ºC or 23.0 ± 0.3 ºC for

15 days were effective for eliminating detectable viable bacteria count of the two *Salmonella spp.* strains studied.

Annex 3

Viral inactivation and removal methods commonly used in human plasma fractions

A3.1 Viral inactivation methods commonly used in human plasma fractions

A3.1.1 Pasteurization

Pasteurization consists of heating continuously a liquid solution at 60 ºC ± 0.5 ºC for 10-11 hours. The treatment denatures viral proteins and inhibits virus replication, affecting both enveloped and non-enveloped viruses (Nowak *et al.*, 1993).

Pasteurization has been used since the late 40s for the inactivation of viruses in human serum albumin (HSA), which was subsequently shown to be effective inactivating Hepatits B Virus (HBV) (Gellis and Neefe, 1948), a highly thermo-resistant virus (Havens, 1946). Although HSA is a thermal stable protein (Ballou *et al.*, 1944), new methods for the albumin stabilization by the addition of non-polar anions were developed to increment its thermal resistance (Ballou *et al.*, 1944; Ballou *et al.*, 1944; Scatchard *et al.*, 1945).

Pasteurization is a very common inactivation procedure used in the HSA production process, after the cold ethanol fractionation, as a final inactivation step and it is also used to treat other plasma fractions as immune globuline solutions, protease inhibitors and coagulation factors. Since these proteins are more thermo-labile than albumin, these solutions have to be treated in presence of citrate, amino acids or sugars as a stabilizers (Horowitz *et al.*, 1985a) to prevent protein denaturation and loss of biological activity. This situation represents an inconvenient because these stabilizers have to be frequently removed after the pasteurization process and, furthermore, the presence of stabilizers may also stabilize pathogenic agents like viruses (Ng and Dobkin, 1985).

Pasteurization of animal plasma without fractionation is not a suitable method due to the denaturalization of proteins when subjected at these temperatures for a prolonged period of time.

A3.1.2 Solvent/detergent treatment

Organic solvent/detergent (SD) mixtures have been effectively used to inactivate lipid enveloped viruses (Horowitz *et al.*, 1985a) because solvent/detergent mixtures cause disruption of the viral lipid envelope. The method consists of the addition of the solvent mixture, commonly Tri (n-butyl) phosphate (TNBP) at 0.3% to 1% with a non-ionic detergent at 1% (Neurath *et al.*, 1972; Horowitz, M.E. Wiebe, *et al.*, 1985). As non-ionic detergents, Tween 80 or Triton X-100 is used. Depending on the detergent used, the mixture must act for 6 hours at 24ºC in the case of Tween80 or 4 hours at 24ºC if Triton X-100 is used. This method achieves the inactivation of enveloped viruses while maintaining the biological activity of plasma proteins, even the most labile fractions (Burnouf, 2007). As a limitation, this method, due to its mode of action, is not effective against non-enveloped viruses and, furthermore, the SD mixture must be removed from the product, which increases the processing time and decreases the product yield (Korneyeva *et al.*, 2002).

This method cannot be applied to animal plasma due to the huge volumes used in this process and the need to remove the SD added to the mixture. In addition, this would not be economically and industrially feasible for animal plasma

A3.1.3 Low pH

It consists of a treatment at pH 4, 30-37 ºC, with the eventual addition of pepsin, during a minimum of 20 hours. Many proteins lose their integrity under acidic conditions; however, immunoglobulins resist this pH without affecting their biological activity. This began to be applied to reduce the aggregation and anti-complementary activity in the fractions of immunoglobulins obtained by cold ethanol fractionation (Morgenthaler, 2001) and it was observed that significantly inactivated enveloped viruses (Omar *et al.*, 1996).

An alternative to the use of SD mixtures is the use of unsaturated fatty acids. Since late 1970s, the properties of fatty acids derivatives as viricides are known (Sands, 1977). In this field, caprylic acid (octanoic acid) was especially important since it was already used as a stabilizer in the albumin heat treatment (Gellis and Neefe, 1948; Yu and Finlayson, 1984) and as a precipitating agent in the production of immunoglobulin G (IgG) (Steinbuch and Audran, 1969; Habeeb and Francis, 1984). Caprylate can be used as a viricidal agent, without causing precipitation of proteins, since it was possible to control the amount of non-ionized acid in the solution to be treated, by strict control of the pH (below 6.5) (Seng and Lundblad, 1990; Lundblad and Seng, 1991).

At this point, caprylic acid was widely used as viricidal agent on enveloped viruses since it was established its effectivity against human immunodeficiency virus type 1 (HIV-1), BVDV (used as hepatitis C virus (HCV) surrogate), PRV (used as hepatitis B virus (HBV) surrogate) in immune globulin intravenous (IGIV) (Korneyeva *et al.*, 2002; Lebing *et al.*, 2003), IgG (Parkkinen *et al.*, 2006) and albumin (Johnston *et al.*, 2003) production processes.

The reduction of pH or addition of fatty acid to industrial animal plasma manufacturing process is not possible due to protein denaturation observed when both methods are applied to whole plasma. These methods have been developed for plasma fractions but not for the whole plasma.

A3.1.4 Dry heat

Another approximation to inactivate viruses from blood derivatives consists of heating protein plasma fractions after its lyophilization. It was stablished that a treatment of dry heat at 80ºC for 72 hours after the lyophilization of a coagulation factor concentrate inactivated HBV, HCV, HIV and Hepatitis A Virus (HAV) (Colvin *et al.*, 1988; Winkelmana *et al.*, 1989). More recently, dry heat at 100°C for 30 minutes was used after lyophilization and good inactivation records were obtained with enveloped viruses (Vesicular Stomatitis Virus (VSV), PRV) (Huangfu *et al.*, 2016, 2018); however, non-enveloped viruses were partially resistant (encephalomyocarditis virus) or completely resistant (Canine parvovirus and human parvovirus B19) (Santagostino *et al.*, 1997; Huangfu *et al.*, 2018) when treating different plasma fractions.

Because dry heat is relatively effective against non-enveloped viruses, it is frequently used as a terminal inactivation step, after other inactivation processes such as SD treatment or as lyophilization (Hart *et al.*, 1994; Huangfu *et al.*, 2018). Nevertheless, it has to be taken into account that viruses may become more stable if they resist the lyophilization process (WHO, 2004). In order to prevent that fact,

residual humidity must be strictly controlled since a higher humidity value reduces the stability of viruses and proteins (Morgenthaler, 2001).

Dry heating of SDP at 80 or 100ºC for different times would not be feasible to be applied to whole plasma without fractionation (due to protein denaturalization and decrease in solubility).

A3.1.5 Vapour heat

Vapour heat is a variant of the dry heat method, consisting of heating the freeze-dried product by steam. Usually a 60°C treatment is applied for 10 hours (Morgenthaler, 2001; Rezvan *et al.*, 2006). Subsequently, another heating step at 80 °C may be applied for 1 hour. This second step has shown greater efficacy in the inactivation of HAV (Barrett *et al.*, 1997). In any case, the application of this second step should not be considered as a redundant safety step (WHO, 2004).

This method of inactivation took special importance in different European countries and USA as a result of the outbreak of HAV in hemophiliac patients who had received transfusions of clotting factors (factor VIII, factor IX) only treated by SD mixtures and purified by ion-exchange chromatography (Vermylen and Peerlinck, 1994; Centers for Disease Control and Prevention (CDC), 1996). In turn, it was observed that these concentrates transmitted human parvovirus B19 to hemophiliac patients (Azzi *et al.*, 1992; Mariani *et al.*, 1993).

Since vapour heating at 60ºC for 10 hours plus 1 hour at 80 ºC was able to inactivate HAV (Barrett *et al.*, 1997), several retrospective studies demonstrated the low risk of viral infection of the clotting concentrates when they are treated by SD and vapor heat (Mannucci *et al.*, 1992; Shapiro *et al.*, 1995).

As indicated for dry heat, the application of vapor heat to SDP at 60 or 80ºC for different times would not be applicable to whole plasma without fractionation due to protein denaturalization and decrease in solubility.

A3.2 Viral removal methods used in human plasma

A3.2.1 Precipitation

Cold ethanol precipitation (Cohn and Strong, 1946; Kistler and Nitschmann, 1962) is the most used method for plasma fractionation. The method consists of progressive processing steps at different concentrations of ethanol, pH, temperature and osmolarity, resulting in the selective precipitation of proteins, mainly albumin and globulins (Burnouf, 2007).

At room temperature and above, ethanol acts as a disinfectant agent; however, ethanol precipitation process uses temperatures between -3 and -5ºC to preserve protein integrity. At these temperatures, ethanol does not contribute substantially to the viral inactivation (WHO, 2004). Nevertheless, it contributes to the viral removal since it partially separates viruses from proteins. Viruses precipitate at early stages of the process, in fractions without therapeutic interest (Morgenthaler, 2001).

It is interesting to note that, when the resultant fractionation precipitates are separated by filtration, especially when filtration adjuvants are used, a greater capacity for virus removal can be obtained (Omar and Morgenthaler, 1997).

This method is applied for plasma fractionation in which the addition of selective amounts of ethanol at controlled temperatures

allows the fractionation of different plasma proteins; however, such methodology cannot be applied to whole plasma because of protein precipitation.

A3.2.2 Chromatography

Chromatography is a physical separation method for the characterization of complex mixtures. The components of the mixture, dissolved in a mobile phase, move with different speed through a stationary phase. This results in the effective separation of the solutes, while these can be identified in terms of their speed of advance.

Some types of chromatography, as ion exchange chromatography and, particularly, affinity chromatography are able to separate enveloped and non-enveloped viruses from proteins (Griffith, 1989; Adcock *et al.*, 1998a; Adcock *et al.*, 1998b; Roberts, 2014; Anwaruzzaman *et al.*, 2015). However, two main limitations are inherent in this technique:

- Since viruses can bind to proteins or resins and chromatography is a very specific process, the results of the virus elimination can be very variable. Such variability is highly influenced by the clone geometry, composition and the flow velocity of the buffers used, aging of resins, etc. (WHO, 2004; Cipriano *et al.*, 2012).
- The tendency of viruses to associate with the resin makes it necessary to properly disinfect it between processes, to avoid the risk of cross contamination. The sanitization of resins is fundamental; however, sometimes it is a challenge since many resins are sensitive to common methods of disinfection (sodium

hydroxide, sodium hypochlorite, hydrochloric acid, high temperatures, etc.) (WHO, 2004).

In consequence, this methodology is not a feasible method to apply to animal plasma due to the volumes used in this industry and the fact that can only be applied to plasma fractions.

A3.2.3 Nanofitration

Nanofiltration is a process consisting of passing a fluid through a semipermeable membrane (between 15 to 40 µm) at a certain pressure. This produces a separation depending on the size of the molecules that can pass through the membrane, obtaining the permeate, the fluid that has passed through the membrane and the retentate, with the components which molecular weight is greater than the pore size of the membrane.

Nanofiltration is the only technique designed specifically to eliminate viruses. During the process, the viruses are removed from the proteins of interest according to their size (Burnouf, 1996; Burnouf and Radosevich, 2003). The virus is retained in the membrane while the proteins of interest cross the membrane and remain in the permeate. Therefore, the mechanism of action of nanofiltration allows removing both enveloped and non-enveloped viruses. Nanofiltration has been used since the early 90s as a redundant biosafety step, with special interest in removing non-enveloped viruses that may resist through applications based on inactivation by SD mixtures or heat (Ng and Dobkin, 1985; Horowitz, 1989; Luban, 1994; Santagostino *et al.*, 1997; Burnouf and Radosevich, 2003; Yokoyama *et al.*, 2004).

One of the main concerns about this technique is its capability to remove small non-enveloped viruses, especially when the protein of interest has a diameter equal or less than the diameter of the virus.

One way to achieve greater elimination rate of these small non-enveloped viruses is aggregation. Viruses often tend to aggregate as a consequence of previous treatments to which the product has been subjected (Burnouf, 1996), or to form aggregates with other molecules, as antibodies (Morgenthaler, 2001). On the other hand, amino acids can be added to the product to produce the aggregation of the virus and increase the elimination capacity of the system (Yokoyama *et al.*, 2004). Another approach is to work with membranes with a larger pore diameter (35 nm) and increase their filtering capacity using larger pore size pre-filters (75 nm) (Troccoli *et al.*, 1998).

Numerous plasma fractions have been treated by nanofiltration, as IVIG and coagulation factors, obtaining good results in both removal of different viruses and protein recovery (Burnouf-Radosevich *et al.*, 1994; Troccoli *et al.*, 1998; Schulman *et al.*, 1999; Johnston *et al.*, 2000; Van Holten *et al.*, 2002; Tomokiyo *et al.*, 2003; Yokoyama *et al.*, 2004; Mazurier *et al.*, 2004; Hongo-Hirasaki *et al.*, 2006, 2011; Bao *et al.*, 2018; Ma *et al.*, 2018).

The nanofiltration method is indicated to remove viruses present in soluble proteins of specific molecular weight in which the protein is recovered in the supernatant and the virus is retained in the retentate. However, due to the amount of different molecular weight of proteins present in the plasma mixture, it is not possible to design a nanofiltration system for whole plasma that would allow passing most of the protein in the permeate and keep the virus or bacteria in the retentate.

In fact, the industry is using the nanofiltration system to eliminate ash and water from fresh plasma and concentrate the plasma in the retentate.

A3.3 Viral inactivation methods used human Fresh Frozen Plasma (FFP)

Currently, the use of FFP has very limited indications. It is used only in case of needing large transfusions, patients with thrombotic thrombocytopenic purpura or in some situations in which the proteins of interest are not available in isolated and/or purified form (Cohen, 1993; Cooper *et al.*, 1994; WHO. Blood Transfusion Safety Team, 2001).

Usually, the FFP is collected from a single donor, individually, in a transfusion bag. The first approach to reduce viral hazards is to submit the plasma bag to quarantine for a stipulated period of time, coinciding with the window period in which the patient could still seroconvert against the viruses initially screened. After that period, the donor is again screened.

The main limitation of this approach is the fact that the method only yields information about the viruses tested, without data regarding other viruses not analyzed (WHO, 2004). This fact makes it essential to use specific inactivation steps that can be carried out on the FFP. One of the most inactivation methods used is the SD treatment. As said before, SD treatment is an effective method to inactivate enveloped viruses. However, SD mixtures have to be removed from the resultant plasma and this kind of treatment implies the pooling of plasma from several donors.

An alternative method to treat the plasma in the bags in which it is stored, is the use of methylene blue (MB, methylthioninium chloride), commonly at concentration of 1 µM, subjected to visible light (45,000 lux) for 1 hour (Lambrecht *et al.*, 1991). This treatment results in nucleic acid damage induced by the photosensitization, due to the affinity of MB for guanine and, furthermore, for the viral core proteins. When the MB is illuminated, an oxygen singlet is produced, which is the catalyst for photooxidation (Tuite and Kelly, 1993; Müller-Breitkreutz and Mohr, 1995; Müller-Breitkreutz *et al.*, 1995).

MB treatment has demonstrated its effectiveness against enveloped viruses (Lambrecht *et al.*, 1991; Mohr *et al.*, 1992a; Wagner *et al.*, 1994; Mohr *et al.*, 1995; Elikaei *et al.*, 2014), but there are discrepancies regarding its effectiveness against non-enveloped viruses because it seems to be effective against members of the genus Adenovirus and Calicivirus, reoviruses and SV40 virus (Mohr *et al.*, 1995), and perhaps also against B19 virus (Mohr *et al.*, 1997). However, it is not active against EMCV, HAV and poliovirus (Mohr *et al.*, 1992a; Wagner *et al.*, 1994; Mohr, 1998).

One of the main advantages of this method of inactivation is to be able to be implemented in the plasma bag, without having to pool the plasma. Initially, since MB is a drug approved for medical use in humans in the case of methemoglobinemia, MB and the compounds derived from its photoreaction are not removed from the plasma (Mohr *et al.*, 1992b; Mohr *et al.*, 1992a; Wieding and Neumeyer, 1992). In addition, there are differences in the treatment using MB between some European countries: in Italy and Spain the treated FFP is administrated without filtration of the compound while other countries, as France, UK, and Austria, the product is filtrated after its treatment.

However, after years of use, some anaphylactic reactions and other allergies were reported after the use of FFP treated with MB (FFP-MB) or other clinical uses of this dye (Dewachter *et al.*, 2005, 2011; Bézu *et al.*, 2011; Nubret *et al.*, 2011). Due to the high number of allergic reactions to FFP-MB in France, and the greater variability of fibrinogen concentration in FFP-MB, the French regulatory authority ANSM eliminated the approval of the use of FFP-MB in February 2012 (New *et al.*, 2012). The German Paul Ehrlich Institute removed the approval of the use of FFP-MB without elimination of MB and its byproducts due to its possible mutagenic effects (Rezvan *et al.*, 2006; Benjamin and McLaughlin, 2012).

This method cannot be applied to industrial animal plasma production due to the huge volumes used in this industry.

Chapter 9

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